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UNIVERSITY OF ALBERTA

THE SIGNIFICANCE OF RETINOL-BINDING PROTEIN IN BREAST CANCER

BY

ALISON JANE RIGBY

A THESIS

SUBMITTED TO THE FACULTY OF

GRADUATE STUDIES & RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF

SCIENCE IN NUTRITION

DEPARTMENT OF FOODS & NUTRITION

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled:

THE SIGNIFICANCE OF RETINOL-BINDING PROTEIN IN BREAST CANCER

Submitted by ALISON JANE RIGBY in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN NUTRITION.

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ABSTRACT

There have been many case control studies in relation to vitamin A status and epithelial cancers. Most of these studies have been based exclusively on levels of serum vitamin A, with healthy subjects used as controls. Few studies have examined the vitamin A status and particularly levels of its carrier proteins, retinol-binding protein (RBP) and prealbumin in the plasma, in patients with benign breast disease and various stages of breast cancer. The present study was conducted to investigate the biochemical status of vitamin A in breast cancer patients, compared with both healthy controls and patients with benign breast disease. In addition, the relationship between plasma vitamin A levels and the factors which are responsible for its metabolic availability, including RBP, prealbumin and zinc were examined. The total study population of 95 females, consisted of 17 patients with metastatic breast cancer; 25 post-operative disease free breast cancer patients; and 28 benign disease breast patients from rural hospitals located in Lamont and Barrhead, Alberta. This total also included 25 healthy control subjects from the Department of Applied Sciences, University of Alberta.

Plasma retinol was found to be significantly lower ($P < 0.05$) in the benign breast disease (2.5 ± 0.2 μmol/L) and the breast cancer patients with disease free status (2.4 ± 0.2 μmol/L), than the healthy controls (3.4 ± 0.3 μmol/L), (mean $\pm$ SEM). More significantly, plasma RBP levels were lower ($P < 0.01$) in the benign breast disease patients (42.9 ± 1.2 mg/L); the post-operative disease free breast cancer patients (45.3 ± 1.4 mg/L); and the

metastatic breast cancer patients (40.5 ± 3.1 mg/L), compared to the healthy controls (53.3 ± 2.4 mg/L), (mean ± SEM). Overall, the advanced breast cancer patients (clinical stage IV), and the deceased metastatic breast cancer patients (7 out of 17 patients), had decreased plasma RBP levels (37.0 ± 3.2 mg/L stage IV versus 43.7 ± 5.0 mg/L stage III, and 35.7 ± 3.4 mg/L deceased patients versus 43.7 ± 4.5 mg/L metastatic breast cancer patients still living), (mean ± SEM).

The biochemical nutritional status of the study population was assessed in addition to other factors such as hepatic function; blood cholesterol levels; body mass index; and adjuvant therapy, which may influence the vitamin A status. The case-control differences in plasma retinol and RBP were not entirely due to a severe protein malnutrition, zinc deficiency, or other confounding variables. The results revealed that it was not only the plasma levels of vitamin A, but also the other factors involved in its metabolism which were affected. RBP was the main significant parameter changed in the benign breast disease and the breast cancer patients. The reduced plasma RBP levels did not, however, appear to be specific only to a state of malignancy. Since plasma RBP concentrations may be used to detect subclinical malnutrition, it appears that RBP can be used as a more sensitive reflection of individual protein status, than the other plasma proteins. Indeed, the possible prognostic significance of RBP cannot be eliminated and this could be substantiated with further research on the recurrence of the disease in the study population.

KEY WORDS **Retinol, retinol-binding protein, metastases, breast cancer, benign breast**

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LIST OF ABBREVIATIONS

A = amp
BMI = body mass index
°C = degrees celsius
dL = deciliter
g = gram
IU = International unit
kDa = kilodalton
kg = kilogram
L = liter
LC = low concentration
m = meter
mA = milliamp
mg = milligram
min = minute
mL = milliliter
n = number
% = percent
P = probability level
ppm = parts per million
RBP = retinol-binding protein
RE = retinol equivalent
rpm = revolutions per minute
SEM = standard error of the mean
µg = microgram
µL = microliter
µmol = micromole
y = year
< = less than
> = greater than

1. INTRODUCTION

The significance of diet and its association with cancer is a complex problem, since neoplastic diseases are attributed to having a multifactorial etiology. It is suggested that environmental factors, including the diet, are responsible for 80-90% of all forms of cancer in humans. Dietary factors alone are estimated to be significant in 30-40% of cancers in males and approximately 60% in females (*Jensen et al., 1988*). Interest is concentrated on the nutritional factors that may either elicit or prevent cancer.

1.1 BREAST CANCER: SCOPE OF THE DISEASE

1.1.1 Breast Cancer Statistics

Cancer is endemic in our population. The incidence rates in North America (and Britain), are appreciably greater than in many other countries (*Brown, 1983*). According to recent statistics, as many as 76 million Americans (30%) now living, will eventually develop cancer (*American Cancer Society, 1991*). The current Surgeon General's Report indicates that cancer is the second leading cause of death in North American (22% total deaths in 1987), costing in the region of $72 billion for health care in 1985 (*DHHS, 1988*).

Breast cancer has the highest (32%) estimated incidence among different types of cancer in females, according to the National Cancer Institute (1991). Breast cancer is the most common malignancy and leading cause of death for women between the ages 35-50 years. However, with improved screening and

surgical techniques, it has been estimated that 77% of breast cancer patients in the United States survive five years after surgery, with 63% living 10 years or more. Currently, every year about 12,300 Canadian women develop breast cancer; it is estimated that approximately 1 in 10 women living in this country will develop breast cancer during her lifetime (*Boring et al., 1991; & Canadian Cancer Society, 1989*).

1.1.2 Risk Factors Associated With Breast Cancer

The stroma of the adult female breast is ordinarily fibrous and rich in collagen with adipose tissue. It has been suggested that in post-menopausal breast cancer, the fibrous stroma may be replaced with uneven adipose tissue to produce nodules, suggesting the presence of a tumor (*Carter, 1990*). See **Appendix #1** for a list of definitions associated with breast cancer. Breast cancer is a disease of the glandular epithelium and it has been thought to result from a hormonally controlled differentiation of stem cells, into mature epithelial cells (*Morabia et al., 1990*). Current knowledge of the mechanisms of differentiation of the mammary gland suggests that both estrogen and progesterone may be involved in breast carcinogenesis, which is supported by the predominance of the disease among females. Previous work has indicated that women with breast cancer were more exposed to estradiol and estrone

(two estrogen fractions), and had a lower ratio of estriol to estradiol and estrone (*MacMohen et al., 1974*).

The "hormonal" breast cancer risk factors for women living in westernized societies have been further described as early age menarche, late age menopause, late age at first term pregnancy and high post-menopausal body weight (*Korenman, 1980; & Tao et al., 1988*). Other researchers have revealed a predominance of increased breast cancer rates at pre-menopausal ages (40-55 years), (*Stevens et al., 1982*). The results of a study in Iceland (*Tryggvadottir et al., 1988*), supported the presence of a genetic factor in the etiology of familial breast cancer. Although the variation in breast cancer rates between countries probably has some genetic component, the marked environmental differences also suggest a role for exogenous factors (*Rohan et al., 1987*).

Cancer involves at least two phases: initiation, the rapid irreversible consequence of damage to cellular macromolecules, such as DNA; and promotion, the accelerated division of the initiated cell which ultimately leads to uncontrollable growth (*Milner, 1986*). Many lifestyle factors, including diet have been linked to the development of cancer. (*Nissinen et al., 1989; & Palmer, 1985*). It is therefore possible that diet could modify the process of carcinogenesis, in either the initiation or promotion phase.

1.2 NUTRITIONAL FACTORS IN THE ETIOLOGY OF BREAST CANCER

1.2.1 Macro-Nutrients

The high kilocaloric content of the diet, especially with regard to the intake of lipids (saturated fat and cholesterol), has been related to the incidence of breast cancer in many studies. (*Kinlen, 1987; Kritchevsky, 1989; & Rose et al., 1990*). Verreault and his associates (1988), investigated the relation of diet in 666 women with a newly diagnosed infiltrating breast carcinoma. After adjustment for total energy intake, age, body-weight and tumor size at diagnosis, an increase in saturated fat intake was related to an increased frequency of node involvement among post-menopausal subjects. Howe et al. (1990), showed a statistically significant positive association between breast cancer risk in post-menopausal women and saturated fat intake.

Patients with breast carcinoma have also been shown to have elevated levels of plasma total lipids, phospholipids and cholesterol as compared to patients with non-breast carcinomas (*Basu et al., 1975*). A significant relationship has been found between dietary fat and breast tumor size in pre-menopausal women, and an inverse relationship with serum cholesterol for post-menopausal women (*Hebert et al., 1989*). It has been proposed that dietary and depot fat can affect circulating levels of steroid hormones, and the binding of these to carrier proteins (*Bennett et al., 1990*). With breast cancer being an

endocrine related cancer, the effect of dietary fat on estrogen metabolism is certainly an issue of concern (*Goldin et al., 1988*).

In addition, there is epidemiological evidence to suggest that differences in post-mastectomy survival rates in Japan and the United States may be attributable to differences in dietary fat intake (*Wynder et al., 1986*). The disease has shown changes of incidence in migrant groups, in that there is a trend for migrant groups to acquire the incidence pattern of the host country (*Miller, 1989*). Such has been the trend of an increasing incidence among younger Japanese women and a higher rate for women living in a westernized society. Correlation studies reported by Willett (1990), comparing national diets with mortality rates for breast cancer, indicate strong associations between intake of meat or animal fat and rates of cancer. However, it seems that both case-control and prospective cohort studies may have limited association between these dietary factors and risk of breast cancer (*Hirohata et al., 1987*).

The major macro-nutrient associated with increased breast cancer risk appears to be dietary fat, but it is difficult to demonstrate whether the effect is due to specific lipid components (*Jensen et al., 1988*). The type of fat consumed, as well as its amount is an important issue. A Canadian study, (*Hislop et al., 1986*), revealed an increased risk of breast cancer with higher consumption levels of whole milk and beef, and a protective effect from fish

consumption. Some oils, including those rich in mono-unsaturates (olive oil), medium chain fatty acids, or omega(w) 3 fatty acids appear to lack tumor-promoting effects, even at high levels (*Wynder et al., 1986*). This may be explained by a direct mechanism involving the dietary modification of membrane structure, or an indirect mechanism causing alterations to the endocrine system and functions; the metabolism of essential fatty acids to biologically active eicosanoids such as prostaglandins, and the suppression of immune response (*Wynder et al., 1986*).

The strong positive correlation between dietary fat intake and human mortality from breast cancer has also been illustrated with the increased incidence of mammary tumors in experimental animals (*Carrol et al., 1985*). Albanes (1987), presented the evidence that caloric restriction inhibits experimental carcinogenesis. Breast cancer induced in rats by chemicals and diets containing 40% of kilocalories as fat, significantly increased the frequency of mammary gland carcinoma, as compared with diets containing 0.5-5% fat (*Williams et al., 1986; & Reddy, 1986*). Polyunsaturated fats have also been found to be more effective promoters of cancer in animal experiments (*Jensen et al., 1988*). This is still controversial, as by contrast another study indicated that an elevation in polyunsaturated fat intake was related to a reduction of breast cancer patients with positive node involvement (*Verreault et al., 1988*). Yet, it would appear that studies on breast tumorigenesis in animals suggest

that diet may be an important factor in the etiology of human breast cancer (*Mettlin, 1986*).

Other studies have found that generally risk of breast cancer is associated inversely with energy and lipid intakes (*Howe et al., 1990; & Knekt et al., 1990)*. Within a study on diet as a risk factor for fibrocystic disease and breast cancer, 68 patients with breast cancer (ages 40-59), participating in the National Breast Screening Study in Montreal, were compared to 340 patients with fibrocystic disease and 343 controls (*Simard et al., 1990*). The cancer patients were significantly heavier and had a higher body mass index than the control subjects, which indicates that body size may be a more important factor than dietary fat intake. No significant differences were found in the use of contraceptives, menopausal hormones, analgesics and tobacco, marital status, number of pregnancies and children, age at menarche, duration of menstrual cycle or age at first pregnancy.

De Waard et al. (1974), and Boyle et al. (1988) associated increased risk of breast cancer with obesity in post-menopausal women. According to Rohan et al. (1987), a relationship between body size and breast cancer could reflect the influence of diet on breast cancer. In this study, involving Benign Proliferative Epithelial Disorders (BPED) of the breast, there is increasing evidence presented against the effect of just dietary energy and fat intake in the etiology of breast cancer. Pre-menopausal subjects have since been shown

to have a positive association between breast cancer and height (*Howe et al., 1990*). It would therefore seem that body mass index (BMI) is the most appropriate way of relating weight and height to breast cancer risk in pre-menopausal and post-menopausal women.

1.2.2 Micro-Nutrients

The ability of vitamins and minerals to prevent cancer has been studied extensively (*Birt, 1986 & 1989; & Leonard et al., 1986*). A consistent protective effect from the intake of fruit and vegetables has been demonstrated; with vitamin C intake having a statistically significant inverse association with breast cancer risk (*Howe et al., 1990*). The prospective study by Wald et al., (1984), showed that lower levels of serum vitamin E were associated with an increased risk of breast cancer. In 1988, Rao et al. conducted a study on 72 rats with azoxymethane-induced tumors, that were maintained on either a low-risk (LR), or high-risk (HR) diet. The diet containing micro-nutrient supplements, a synthetic analogue of vitamin A (13-cis-retinoic acid, 50 μg/kg diet), vitamin E (alpha-tocopherol acetate, 5g/kg diet), and selenium (sodium selenium, 4 ppm), was considered to be the LR diet. The protective effect of the LR diet in reducing tumor incidence was dramatic, with an 86% reduction in the incidence of colonic adenocarcinomas compared to the HR dietary groups. It seemed that by combining several chemopreventative dietary manipulations

into a "low-risk" diet, a significant decrease in tumor incidence could be achieved; without the need for the extreme dietary levels, that had been utilized in previous studies.

Geographic studies of the correlation between per capita intake of selenium and its serum level, have suggested that selenium reduces the risk of carcinogenesis (*Diplock 1990; & Jensen et al., 1988*). Women with breast cancer have been found to have lower serum concentrations of selenium compared to age-matched control subjects (*Husami et al., 1986*). *Koskinen et al.* (1987), looked at the differences in serum levels of selenium, along with vitamins A and E, and cholesterol in Finnish and Japanese post-menopausal women, considered to be high and low breast cancer risk populations, respectively. Both groups had identical serum vitamin A levels, but the other serum factors differed significantly between the groups. A poorer selenium status (and higher serum vitamin E and cholesterol), was found in the Finnish women compared with women in Japan, where rice and seafood rich in selenium are consumed. Diet supplemented with selenium has also been reported to inhibit mammary gland and colon carcinogenesis in several experimental studies (*Birt, 1989*). These experimental results are in agreement with the results from human studies (*Salonen et al., 1985*).

Unlike selenium, zinc has been implicated in tumor induction as demonstrated with retarded tumor growth in zinc deficient animals (*Birt et al.,*

1989). While zinc appears to be an essential nutrient for neoplastic growth in animal tumor models (*Kurzer et al., 1986*), its deficiency has been reported in patients with laryngeal cancer (*Drozdz et al., 1989*). Epidemiologically, oesophageal cancer has been reported to be associated with reduced zinc levels in serum, hair and tissue (*Barch, 1989*). A zinc deficiency resulting in the inhibition of protein synthesis, may also affect tissue availability of other micro-nutrients that rely on serum transport proteins, such as vitamin A. Trace elements particularly zinc as well as vitamins, appear to play important roles in the maintenance of immunocompetence and are of significance in the dietary prevention of certain cancers (*Good et al., 1990*).

Experimental results in animal models consistently suggest an inhibitory role for vitamin A in tumor initiation and promotion (*Mehta et al., 1987*). Epidemiological studies too, suggest that low pro-vitamin A carotene intake and low serum retinol may be associated with an increased risk of cancer (*Willet et al., 1984*). The impact of vitamin A and ß-carotene in cancer and specifically breast cancer, is explored further in the following sections.

1.3 OVERVIEW OF VITAMIN A METABOLISM

1.3.1 Absorption, Regulation and Transportation of Vitamin A

The term "vitamin A" refers to retinol (preformed vitamin A) and its synthetic analogues, or to carotenoids (pro-vitamin A), which can be converted to retinol in the body (*Hennekens et al., 1986*). It is estimated that the overall absorption efficiency of dietary vitamin A from animal foods is 80-90% and ß-carotene from plant foods is 50-60%, with less efficient absorption at higher doses (*Olson, 1984*). Major dietary sources of retinol include dairy products, liver and fish; with the choice items for ß-carotene being carrots, green leafy vegetables and fruits such as papaya and mango. Currently, 12 μg of mixed carotenoids in a typical food, or 6 μg of ß-carotene, is considered equivalent to 1 μg of retinol (*Olson, 1984*).

Maximum ß-carotene absorption requires its release from endogenous proteins, presence of dietary fats and secreted bile acids (*Linder, 1985*). During the absorption process in the intestine, ß-carotene is cleaved into 2 retinal (aldehyde) units, which are incorporated into chylomicrons and transported, via the blood and lymph to the liver. Excess vitamin A can be stored in the liver as fatty acid esters of retinol (palmityl) and excess ß-carotene (which has not been converted into retinol), can be stored in fatty tissues and carried in the low-density lipoproteins (*Wolf, 1980*). It is recognized that the liver contains an average of 100-300 μg retinol/g of wet tissue, taking some 220 days to

reduce serum levels of retinol to subnormal levels, or about 20 days for depletion when concentrations are below 20 μg retinol/g of wet tissue (*Riboli et al., 1987*). The storage capacity of the liver contains over 90% of total body stores, with the plasma content representing only about 1% of the total reserves of this vitamin (*Ostrowski et al., 1989*).

Plasma levels of retinol (alcohol) are regulated homeostatically and so unaffected by intake, except in a state of severe deficiency or toxicity. However, serum carotene levels are highly variable and usually reflect dietary intake. Thus, giving 25,000 IU of retinol palmitate had no effect on plasma retinol or carotenoids, compared to daily doses of 30 mg ß-carotene, which tripled the levels of carotenoids, but did not affect retinol or alpha-tocopherol (*Willett et al., 1983*). The influence of dietary fats and vitamin E on plasma and hepatic vitamin A and ß-carotene levels have been studied in the animal model (*Alam et al., 1990*). Plasma vitamin A levels were not altered by any of the dietary lipids or an excess of vitamin E, whereas hepatic vitamin A concentrations were affected by the type of dietary fat, being highest in the rats fed a coconut oil diet and lowest with menhaden oil (rich in w-3 fatty acids).

Retinol is released from the liver to meet the needs of the body. To facilitate transportation from the liver via the blood to target tissues, retinol combines with retinol-binding protein, which is synthesized in the liver. This

then combines with prealbumin (transthyretin or TTR), in the plasma to form a 1:1:1 complex, preventing any loss from glomerular filtration. Receptors at the cell surface are then presumed to be involved with the uptake of retinol (*Goodman, 1984*), and transfer to specific intracellular retinoid binding proteins (CRBP), (*Chytil et al., 1982*). Retinol can be oxidized to retinaldehyde and onto retinoic acid, used for metabolism and growth, but at this end-point it is irreversible and these compounds cannot be stored.

1.3.2 Factors Affecting Retinol-Binding Protein Levels

Extraneous factors that may affect plasma retinol levels include hormone levels of estrogens (either endogenous or those used in contraceptive agents). Estrogen increases plasma retinol and RBP, as a result of increased mobilization of vitamin A from the liver (*Gibson, 1990*). Stress is another factor which is known to reduce plasma retinol levels, as a result of decreased synthesis and secretion of RBP. On the other hand, the glucocorticoid hormones may accelerate vitamin A mobilization causing the production of RBP (*Smith et al., 1979*).

Protein-energy malnutrition is known to be an important factor, which decreases RBP production (*Gibson, 1990*). This would cause an impairment of the hepatic release of vitamin A, resulting in decreased plasma retinol levels. The role of retinol in the secretion of RBP is of considerable importance, as

RBP secretion is specifically blocked in the absence of retinol (*Smith et al., 1979*). This has been demonstrated with the administration of retinol to vitamin A deficient rats, stimulating the rapid secretion of RBP from the liver into the plasma, eventually causing plasma RBP levels to fall and liver RBP levels to rise (*Ganguly, 1989*). The block in RBP secretion is highly specific for this protein and a vitamin A deficiency would have no effect on plasma levels of prealbumin. It seems, therefore, that the plasma concentration of RBP is regulated by the protein and vitamin A status of the individual. The plasma RBP would be very low for those persons lacking vitamin A, except in patients with an impaired glomerular filtration rate (*Rask et al., 1980*). In chronic renal disease, the half-life of RBP is increased (*Gibson, 1990*). Apart from cases of deficiency or excess, retinol levels in the plasma alone are not a significant indicator of vitamin A status (*Olson, 1984*).

The mobilization of vitamin A from the liver is also known to be impaired by zinc deficiency. Zinc plays an important role in protein metabolism in humans and is necessary for the maintenance of normal levels of certain transport proteins (*Bates et al., 1981*). Animal research has shown (*Lonnerda, 1988*), that zinc-deficient animals have lower plasma vitamin A levels, in parallel with reduced levels of plasma RBP. Hence a zinc deficiency would decrease plasma retinol levels via its role in the synthesis of RBP. The fall in

plasma RBP levels during deficiencies of vitamin A or zinc, is in response to a reduced demand for the vitamin A transport protein (*Gibson, 1990*).

Some of the other factors affecting concentrations of RBP are similar to those for other serum proteins, such as albumin and transferrin. Concentrations of RBP would also decrease in hepatic diseases, as a result of interference with the storage and synthesis of RBP in the liver.

1.3.3 Function and Proposed Mechanism of Vitamin A

Vitamin A has been associated with vision, reproduction and maintenance of differentiated epithelia and mucus secretion (*Goodman, 1984*). Daily requirement is calculated on the basis of retinol equivalents, as 4000/5000 IU (or 800/1000 μg RE) of retinol for women/men, respectively (*Linder, 1985*). An additional 1000 IU is recommended during pregnancy and lactation. Vitamin A deficiency is accompanied by morphological changes in the epithelial cells, characterized by metaplasia and hyperkeratosis, which could increase susceptibility to carcinogenesis (*Yamanaka, 1987*). Since prolonged use of vitamin A at high doses may be toxic, analogues of 13-cis retinoic acid (which is not stored), are being developed for use in epithelial cells against cancer (*Shealy, 1989*).

It is suggested that retinoids act by inhibiting the "promotion" phase of carcinogenesis and have an effect on cell proliferation and differentiation

(*Purfilo, 1987*). Other effects of retinoids, related to the inhibition of cancer, include the inhibition of carcinogen binding to DNA; effect on the cell cycle and division; alteration of membrane and surface recognition; and possibly immune stimulation (*Birt, 1989; & Watson, 1986*). The "Free Radical Theory of Carcinogenesis" suggests that genetic modification caused by free radicals formed by oxidation of carcinogens, leads to transformed cells (*Milner, 1986*). These free radicals being the highly reactive molecules which can cause tissue damage by reacting with polyunsaturated fatty acids in cellular membranes, nucleotides in DNA, and critical sulfhydryl bonds in proteins (*Machlin et al., 1987*). Study results (*D'Aquino et al., 1989*), support the possibility that vitamin A may play a role in protecting lipid membranes against free radical (thiyl, derived from glutathione) mediated damage.

ß-Carotene is a highly active free radical scavenger, therefore protecting cellular structures from oxidative damage (*Stahelin, 1988*). This is supported by epidemiological studies, which consistently find an inverse relationship between consumption of carotene rich fruit and vegetables and cancer risk (*Hennekens et al., 1986*). The anti-oxidative property of ß-carotene is of significance, relative to vitamin A, which cannot quench singlet oxygen (*Good et al., 1990*). By scavenging free radicals as they arise in the body or by suppressing the expression of neoplasia in carcinogen-induced cells (*Wattenberg, 1983*), it would appear that retinol and ß-carotene have roles to play in the

prevention of cancer. Carcinogenesis may indeed alter the metabolism of fat soluble vitamins, specifically in various forms of cancer, and these alterations of vitamin metabolism could be in some way involved in the carcinogenic process (*Rougereau et al., 1987*). Ultimately, according to Sporn et al., 1983, the molecular mechanism for the action of retinoids in the control of differentiation and carcinogenesis is converging on one of the central problems in medical science, which is the control of gene expression.

1.4 EPIDEMIOLOGICAL STUDIES CONCERNING VITAMIN A AND CANCER

1.4.1 The Significance of Vitamin A

There is extensive literature pertaining to the relationship between vitamin A and cancer. Epidemiological studies have provided evidence that there is an inverse relationship between risk of cancer of epithelial cell origin and either vitamin A (and pro-vitamin A) intake, or serum vitamin A levels. Knekt and associates (1990), examined serum vitamin A and subsequent risk of cancer, as part of the Finnish Mobile Clinic Health Examination Survey. According to this longitudinal study, individuals with higher serum retinol and RBP levels had a lower risk of breast cancer. Likewise, plasma vitamin A is lower in cancer patients, particularly with cancer of the pancreas and liver (*Husami et al., 1986*). In earlier studies, serum vitamin A concentrations were

measured in lung cancer patients and found to be significantly lower than controls (*Atukorala et al., 1979*). The results from Basu et al. (1976), indicated that the plasma vitamin A levels of patients with bronchial carcinoma varied with the histological type of tumor (with low levels in the squamous type carcinoma).

Vitamin A levels were measured in the stored serum samples from 86 apparently healthy males who were subsequently notified as having cancer and in the stored samples from 172 controls, who did not develop cancer (*Wald et al., 1980*). The mean retinol levels were found to be significantly lower in those destined to develop lung cancer, compared to those who remained disease free; this was independent of age, smoking habits and serum cholesterol level. Consumption of pre-formed vitamin A (and ß-carotene) were significantly and independently associated with epidermoid lung cancer (*Dartigues et al., 1990*), illustrating that vitamin A may have a distinct and important protective effect.

Research results have associated decreased intake of dietary vitamin A with an elevated risk of breast cancer (*Graham et al., 1982; & Mettlin, 1984*). A case-control study of the role of diet in the cause of breast cancer was conducted in Greece (*Katsouyanni et al., 1988*). Cases reported a significantly less frequent consumption of vitamin A, after controlling for total caloric intake, potential external confounding variables and other nutrients associated with breast cancer risk. Other case-control studies (*Hislop et al., 1990 & Rohan et*

al., 1990), illustrated a decreased risk of benign proliferative epithelial disorders (BPED) of the breast, with higher intake of retinol and ß-carotene.

There are, however, other studies, which have failed to show any significant association between dietary retinol and risk of breast cancer (*Marubini et al., 1988; & Gerber et al., 1988*). Likewise, other investigators have found no association between serum levels of retinol and cancer (*Coates et al., 1988; LeGardeur et al., 1990; Paganini-Hill et al., 1987; & Willet et al., 1984*). A prospective (8 year) study of the relationship of serum vitamins A and E, and risk of breast cancer, was conducted with 5,086 women resident in Guernsey (*Russell et al., 1988*). No relationship was found between serum vitamin A levels and the subsequent development of breast cancer, although serum retinol binding protein (RBP) and vitamin A concentrations were highly correlated in both the pre-cancer cases and controls.

There have been a few studies suggesting that serum RBP may be of significance in cancer. In a case control study (*Atukorala et al., 1979*), it was reported that patients with lung cancer were associated with low serum retinol levels, in parallel with low levels of RBP and zinc. In patients with colorectal cancer, lower plasma levels of both retinol and RBP have also been reported (*Basu et al., 1985*). It was of interest that RBP values were even lower in 12 post-operative disease free patients who subsequently had cancer recurrence and lowest for 2 patients who died during the follow-up period. Significant

reductions of serum RBP, prealbumin, and zinc levels have also been reported in pancreatic cancer patients, compared with controls (*Fabris et al., 1984*).

For breast cancer, women with lower plasma RBP during the course of chemotherapy had tumor recurrence earlier than those who had higher RBP levels (*Mehta et al., 1987*). In addition to plasma RBP, prealbumin, vitamin A and ß-carotene levels were assessed in pre-menopausal women with node-positive breast carcinoma receiving adjuvant chemotherapy. Results of this study revealed that significantly lower RBP levels were associated with early tumor recurrence. Patients who maintained a disease free status for 12 months or longer had significantly higher plasma RBP levels, compared with those who had tumor metastasis at distant sites. Breast cancer patients with a prior history of benign breast disease also had significantly lower RBP levels compared with healthy, pre-menopausal women. This study implies that lower plasma RBP may alter the regulated vitamin A delivery to tissues, in spite of adequate vitamin A intake.

1.4.2 Evidence for Beta-Carotene (Pro-Vitamin A)

An association between ß-carotene and incidence of lung cancer has been well recognized (*Nomura et al., 1985*), as also underlined from a study by Menkes et al. (1986). The relationship of serum vitamin A, ß-carotene, vitamin E and selenium to the risk of lung cancer were studied and from this a strong

inverse association between serum ß-carotene and the risk of squamous-cell carcinoma of the lung was observed. In a study by Wald et al. (1988), the concentration of ß-carotene was measured in the stored serum samples from 271 males that had cancer and 553 healthy controls, matched for age, smoking history and duration of storage of the serum samples. The mean ß-carotene levels of the cancer subjects were significantly lower than the matched controls. The subjects in the top two quintiles of serum ß-carotene had only about 60% of the risk of developing cancer, compared with the bottom quintile. Stahelin et al. (1984), showed that ß-carotene levels in plasma were significantly lower in cancer cases of the lung and stomach than in controls. The plasma concentration of ß-carotene was also inversely associated with cancer mortality in the prospective Basel study (*Stahelin, 1988*), which further confirms the inverse association of ß-carotene with lung cancer. In a multivariate analysis of data from 64 lung cancer cases, the mean serum levels of ß-carotene along with vitamin A, were also found to be significantly lower than 63 randomly selected healthy controls (*Kune et al., 1989*).

It appears that cigarette smokers have reduced serum levels of ß-carotene (*Comstock et al., 1987*), and this significant association between ß-carotene and lung cancer, may indicate that a low serum ß-carotene level is an important predictor for increased lung cancer risk (*Nomura et al., 1985*). In the Multiple Risk Factor Intervention Trial (MRFIT), both total carotenoids and ß-

carotene levels were lower in 66 lung cancer cases than their matched controls (*Connett et al., 1989*). Carotene levels in both sexes have been shown to be significantly lower in subjects with gastric dysplasia than in subjects with normal mucosa (*Haenszel et al., 1985*). According to Gey et al. (1987), the risk of fatal lung disease and stomach cancer may be lowest at ß-carotene plasma levels above 0.4 μmol/L (215μg/L). It is suggested that ß-carotene should no longer be treated as an optional pro-vitamin A, but be given the status of an essential micronutrient. This is especially important because ß-carotene is believed to be an anti-oxidant (*Good et al., 1990*), which is a function independent of its pro-vitamin A activity.

Subjects with breast cancer have also been shown to have lower concentrations of ß-carotene than controls (*Potischman et al., 1990*). Many dietary epidemiological studies have shown an inverse relationship between dietary intake of ß-carotene and cancer risk (*Ohno et al., 1988; Rohan et al., 1988; Slattery et al., 1989; & Verreault et al., 1989*). Among 645 female controls who participated in the Canadian National Breast Screening Study, a high intake of carotenoids and fibre was associated with a reduction in the extent of high risk densities on the mammogram (*Brisson et al., 1989*). It is possible that carotenoids may play a protective role with respect to breast cancer (*Patton et al., 1990*), which promotes a need for further investigation of this nutrient.

1.5 ADDITIONAL EXPERIMENTAL AND CLINICAL STUDIES

1.5.1 Experimental Studies Involving Vitamin A and Cancer

In experimental studies, a deficit of vitamin A appears to increase susceptibility of animals to the development of chemically induced tumors (*National Research Council, 1982*). Vitamin A and related retinoids have additionally been shown to inhibit carcinogenesis in a variety of organs in animal models (*Wargovich et al., 1988*). This suggests that physiological levels of these natural substances may exert a protective effect against cancer (*Band et al., 1989*). However, controversial evidence does exist and in some experiments retinoids have enhanced carcinogenesis (*Birt, 1989; & Rao et al., 1988*). The lowest tumor incidence has been found in rats fed diets high in vitamin A and low in fat. However, an increase in the level of vitamin A above 10 μg/g feed had no further beneficial effect (*Newberne et al., 1990*).

Both ß-carotene and alpha-tocopherol have been shown to be capable of regressing established epidermoid carcinomas of the hamster buccal pouch, when injected locally into the tumor site. Yet, neither has been shown to be effective in regressing cancer when administered by the oral route (*Shklar et al., 1989*). In a further study, when animals were loaded with carotenoid supplementation one month before a carcinogenic induction procedure, cancer prevention was observed in 60-100% of the test animals (*Santamaria et al., 1990*). Similarly, 15 patients were supplemented with ß-carotene to prevent

cancer recurrence after radical removal of the primary neoplasia in organs such as lung, breast and colon, from 1980-1989. A longer than expected disease free interval was found by Santamaria and researchers (1990).

1.5.2 Clinical Trial Studies for Vitamin A and β-Carotene

One of the direct methods to determine whether retinoids have a beneficial effect is through large, carefully conducted randomized trials (*Hennekens et al., 1986*). Several such studies are currently under way and should provide the evidence for future dietary policy and practice. The U.S. National Cancer Institute has initiated some of these prospective trials giving subjects supplements of ß-carotene in the range of 15-50 mg/day (approximately 10-30 times the usual intake level). The safety of this level of intake has been well documented and used in the past to treat photosensitivity diseases (*Bendich, 1988*).

The ß-carotene trial conducted at the Tom Baker Cancer Centre in Calgary, was an attempt to provide insight into the prolonged consumption of ß-carotene and the occurrence of cancers among patients with recent non-melanoma skin cancer (*Siu et al., 1991*). Approximately 1,300 subjects were expected to participate and the subjects randomly assigned to 25 mg/day regimen of either ß-carotene or placebo. Linked with the promotion of clinical studies, Santamaria et al. 1989, suggested that any epithelial cancer after

radical surgery, can be chemo-prevented with supplemental carotenoids. The possibility of using ß-carotene in the form of sweet potatoes or palm oil, which could contain an economical source of ß-carotene for developing countries, has been explored by Stich et al. (1989).

A clinical study by Band et al. (1984), included 12 patients with benign breast disease (BBD) who were treated with 150,000 IU of vitamin A, taken orally every day. All patients were symptomatic and had measurable breast masses. After 3 months of treatment, complete or partial responses were observed in 5 patients and marked pain reduction was observed in 9 patients. The side effects were generally mild, with no hepatotoxity observed, which may suggest further investigations into the chemopreventative role of either vitamin A or retinoids for women with BBD, who are at a high risk of developing breast cancer.

Several completed chemopreventative studies indicate that certain micronutrients can prevent neoplastic growth (*Dewys et al., 1986*), although the follow-up period has been described as being too short for most nutritional intervention studies, to determine whether prevention strategies are effective (*Holm et al., 1989 & 1990*). According to the National Research Council (NRC, Committee on Diet, Nutrition and Cancer), the toxicity of vitamin A in doses exceeding those required for optimum nutrition, and the difficulty of epidemiological studies to always distinguish the effects of carotenes from those

of vitamin A, argue against an excessive vitamin A intake by the use of supplements. It is not possible to specify any dosage of vitamin A that would have an anticarcinogenic effect at present, and large doses may be toxic *(Bendich et al., 1989; & Jensen et al., 1988)*. It is stressed that further indepth trials need to be done to find out which vitamin A analogues and levels of dietary intake affect which type and stage of cancer (*Watson, 1986 et al., 1986*).

1.5.3 Further Studies Related to Vitamin A and Cancer

There is also a known statistical correlation between retinol and cholesterol concentrations with cancer mortality, which may reflect a relationship between their carrier proteins, RBP and low-density lipoprotein (*Reddy et al., 1986*). In a study examining the relationship of diet and altered cholesterol metabolism with carcinoma, bile acid excretion was significantly less in female cancer patients and a significantly lower intake of retinol and vitamin A was demonstrated in these patients (*Tomkin et al., 1986*). Interrelationships among circulating levels of cholesterol, vitamin A, and selected transport proteins, as well as other nutritional variables were examined in a large population of hospitalized cancer and non-cancer patients, in order to clarify a relationship between serum cholesterol and vitamin A (*Flaim et al., 1986*). Serum cholesterol and vitamin A levels were positively correlated in both groups. The results suggested that serum transport protein levels and

nutritional status are important factors that lead to an association between serum cholesterol and vitamin A. Both deficiency and excess of vitamin A are known to alter the composition of tissue lipids. It has been proposed that some of these changes in fatty acid composition may be due to the retinoid-induced modulation in the activity of fatty acid desaturases (*Alam et al., 1984 & 1985*). This may lead to future studies that compare the lipid profiles and blood essential fatty acid (EFA) levels of benign and malignant breast disease, with control samples.

In the meantime, diets recommended by leading government agencies include fruits and vegetables rich in vitamin A and ß-carotene. The NRC (*Palmer, 1986*), recommends that fat should constitute 30% or less of energy intake and with an increased intake of fruits, vegetables and complex carbohydrates, this should equate to 25-30g of fibre, daily. Analysis of the nutritional recommendations from the American Cancer Society, shows that anyone following these guidelines could be eating in the region of 5-6 mg of ß-carotene per day, compared to the average intake of 1.5 mg (250 RE) of ß-carotene, based on the U.S. Department of Agriculture Food Intake Survey. It is difficult to recommend an "optimum" diet for cancer prevention and it would be preferable that a balanced diet be initiated from early life (*Higginson, 1983*). Dietary moderation appears to be expedient, along with a high consumption of fresh, raw vegetables and fruits.

1.6 CHAPTER CONCLUSION AND OBJECTIVES OF THE PRESENT STUDY

There appears to be a substantial amount of evidence suggesting that a low vitamin A status is significantly correlated with elevated cancer risk. Some of the studies presented illustrate that it is the carrier protein of vitamin A, retinol-binding protein (RBP), that is subnormal in the plasma of patients with established cancer (*Basu et al., 1982 & 1985*). Lower RBP levels have also been associated with tumor recurrence (*Mehta et al., 1987; & Basu et al., 1988 & 1989*). Further work in this area would be valuable in exploring the prognostic significance of this protein in cancer.

Advanced cancer is often connected with anorexia, weight loss and cachexia, and lower serum retinol can be linked with protein-energy imbalance (*Tyler et al., 1984*). The concentrations of RBP and prealbumin in serum are independently regulated and it has been suggested that for a true vitamin A deficiency, serum prealbumin levels remain normal, whilst RBP levels are low. If low levels of retinol are associated with subnormal circulating levels of RBP, it could be proposed that RBP would be a valuable tumor marker. Retinol binding protein is highly sensitive to malnutrition, especially deficiencies of protein, zinc and vitamin A (*Ingenbleek et al., 1975; Smith et al., 1979; & Smith, 1980*). It has been suggested that low levels of zinc reduce the synthesis of RBP and consequently reduce the mobilization of vitamin A from the liver

stores (*Drozdz et al., 1989*). It is therefore possible that secondary factors, rather than the cancer itself, are responsible for the lowered plasma RBP levels in patients with established cancer and those who are destined to have a recurrence.

There are still gaps in the existing knowledge of the etiology of breast cancer and specifically the prognostic and possible diagnostic significance of RBP. It is hypothesized that lower plasma RBP levels correlate with poorer prognosis in cancer. This hypothesis will be tested in a prospective clinical study, using breast cancer as a model. In patients with breast cancer in this study, there will be a focus on two periods in the course of the illness: a) when patients have undergone surgical resection with curative intent; and b) when metastatic disease has been diagnosed or patients have recurrence. In addition to this, a healthy control group and patients with benign breast disease have been included.

The Objectives of the Study Were As Follows:

1. To determine the biochemical status of vitamin A in the patients with metastatic breast cancer; post-operative disease free breast cancer; benign breast disease; and healthy control subjects.

2. To examine in the study population, the relationship between the plasma vitamin A levels and the factors which are responsible for its metabolic availability.

3. To assess the biochemical nutritional status of the study population, in relation to total protein and prealbumin levels in the plasma.

2. *METHODOLOGY*

2.1 STUDY POPULATION

2.1.1 Controls and Patients

The study population consisted of 95 individuals, including 25 healthy post-menopausal female controls, whose plasma samples were obtained from the Department of Applied Sciences, Medicine Bone Research Group, University of Alberta. The original aim of obtaining these subjects was for a calcium and bone density study, but separate plasma samples were collected and stored for the sole purpose of this study. Most other studies concentrate on a healthy control group, whereas this study is unique in its additional focus on the association between patients with benign and malignant disease.

Female patients were recruited from the breast clinics of Archer Memorial Hospital, Lamont and Barrhead General Hospital, Barrhead, Alberta. The local hospitals of Lamont and Barrhead are each geographically responsible for a total population of approximately 7-8 thousand and 25 thousand persons, respectively. Women from Northern Alberta come to these hospitals for routine breast examinations at the clinics, before diagnosis. Some are diagnosed as benign and others as malignant, so would continue to attend the hospitals for follow-up. An advantage of obtaining the plasma samples from the rural hospitals was the access to patients with benign breast disease at the onset of possible complications, while not undergoing any kind of treatment.

The study began in 1989 and arrangements were made with the hospitals (**Appendix #2**), to obtain additional information from the medical records regarding pre-menopausal or post-menopausal age of each individual; details of family history of breast cancer; normality of liver function tests and cholesterol levels in the breast cancer patients; with date and type of surgery and adjuvant therapy (**Appendices #3-7**). Prior to commencement of the study, approval was obtained from the University of Alberta human ethics committee (copy **Appendix #8**); and individual consent from those participating in the study.

2.1.2 Sample Size

Using standard deviations and the normal values for both RBP and vitamin A (*Basu et al., 1989*), a statistically meaningful sample size for each category of the study population was determined. (See calculation details in **Appendix #9**). According to this calculation, a minimum of 16 subjects in each group was required, and therefore, the sample size of 17-28 for each category in the present study should be statistically acceptable.

In addition to the 25 control subjects, plasma samples from patients with breast cancer were obtained at random from the two hospitals and divided into two categories: 17 patients with advanced breast cancer, with metastases (7 now deceased); and 25 patients with early stage breast cancer before surgery

and diagnosed as disease free. Disease free, being defined as having undergone surgery and at the time of blood collection being free from the presence of a tumor. Additionally, at random from both hospitals, 28 plasma samples from patients with benign breast disease were obtained (see **Table 2-1**). Breast tissue samples from Archer Memorial Hospital, were obtained concurrently with the plasma samples from 4 early-stage breast cancer patients (now disease free), and 7 benign breast disease patients.

2.1.3 Age and Body Weight

At the time of the study, the mean age (in years ± SEM) of the control group was 74.1 (± 0.8), compared to 72.3 (± 3.7) for the metastatic breast cancer patients; 67.5 (±2.9) for the post-operative disease free breast cancer patients; and 47.7 (± 1.8) for the benign breast disease patients (**Table 2-2**). Some anthropometric data of the study population is also presented in **Table 2.2**, with the mean weight (± SEM) of the control group being 66.6 kg (± 1.9), compared to 69.3 kg (± 3.4) for the metastatic breast cancer patients; 68.5 kg (± 2.7) for the post-operative disease free breast cancer patients; and 63.1 kg (± 2.3) for the benign breast disease patients. Individual heights were also determined to calculate body mass index.

Although the study population was obtained at random, the benign breast disease patients were significantly younger, with a lower BMI (24.0 ± 0.8

TABLE 2-1

Summary of clinical data of benign breast disease and post-operative breast cancer patients

CLINICAL PARAMETERS	BENIGN BREAST DISEASE	n	POST-OPERATIVE DISEASE FREE BREAST CANCER	n	METASTATIC BREAST CANCER	n
STAGE OF CARCINOMA (before surgery)	No Carcinoma	28	Early Breast Cancer	25	Advanced Breast Cancer	17
			(Clinical Stage I)	12	(Clinical Stage III)	9
			(Clinical Stage II)	13	(Clinical Stage IV)	8
TYPE OF DISEASE (before surgery)	Fibrocystic Disease	24	Infiltrating Duct Carcinoma	17	Infiltrating Duct Carcinoma	15
	Fibroadenoma	3				
	Lipoma	1	Infiltrating Lobular Carcinoma	3	Advanced Type* Carcinoma	2
			Non-Infiltrating Duct Carcinoma*	2	(Now Deceased)	7
			Medullary Carcinoma	2		
			Papillary Carcinoma	1		
SURGERY (additional to mammogram)	Segmental Mastectomy	2	Total Mastectomy	8	Total Mastectomy	5
	Excisional Biopsy	15	Modified Radical Mastectomy	9	Modified Radical Mastectomy	7
	Incisional Biopsy	1				
	Open Biopsy•	3	Segmental Mastectomy	7 (+2)	Segmental Mastectomy	3 (+3)
	Needle Biopsy	5 (+ 7)•	Excisional Biopsy	(+5)	Excisional Biopsy	(+4)
	No Surgery	2	Incisional Biopsy	1	Needle Biopsy	(+1)
					No Surgery	2

n = number of subjects.

• numbers in parenthesis account for more than one type of surgery employed with individual patients.

* unknown type of carcinoma.

• unknown type of open biopsy; either excisional or incisional.

TABLE 2-2

Some anthropometric data of the study population[1,2]

PARAMETERS	CONTROLS (n = 25)	BENIGN BREAST DISEASE (n = 28)	POST-OPERATIVE DISEASE FREE BREAST CANCER (n = 25)	METASTATIC BREAST CANCER (n = 17)
AGE (y)	74.1 ± 0.8[a*]	47.7 ± 1.8[b*]	67.5 ± 2.9[a*]	72.3 ± 3.7[a*]
WEIGHT (kg)	66.0 ± 1.9[a]	63.1 ± 2.3[a]	68.5 ± 2.7[a]	69.3 ± 3.4[a]
HEIGHT (m)	1.56 ± 0.03[b]	1.62 ± 0.01[a]	1.59 ± 0.01[ab]	1.60 ± 0.02[ab]
BMI (kg/m^2)	26.5 ± 0.7[ab]	24.0 ± 0.8[b]	26.9 ± 1.1[a]	27.4 ± 1.4[a]

[1]Values are means ± SEM for the number of subjects shown in parenthesis.

[2]In each row, values not sharing a common superscript letter are significantly different at $P < 0.05$, * $P < 0.01$.

versus 26.5 ± 0.7 for the controls), (mean ± SEM). Typically it is the younger age group of women that report to the hospitals with benign breast disease, whereas these parameters are all comparable for the healthy controls; post-operative disease free breast cancer patients; and metastatic breast cancer patients, all of whom were older (**Table 2.2**). Since age has little effect on plasma vitamin A levels (*Comstock et al., 1987*), it was felt that a separate control group age-matched with the patients with benign breast disease, was not necessary.

2.1.4 Sample Collection

Over two years, the separated plasma samples were frozen packed in dry ice and sent from the hospitals to the Department of Foods and Nutrition, University of Alberta. Similarly the tissue samples were forwarded in sealed vacutainers (also packed in dry-ice), and both stored immediately in the freezer (at -20°C). In addition, a patient clinical history form (**Appendix #10**), was completed and submitted by the surgeons. The control plasma samples were collected directly from the Medicine Bone Research Group. Since vitamin A is extremely light sensitive (*Olson, 1984*), the collection tubes (5-10 mL), were wrapped with aluminum foil during transportation and final storage at -20°C.

The effect of long-term storage (in this case between 1 - 2 years), on the stability of light-sensitive vitamin A could be a possible concern. Prospective

studies (*Mejia et al., 1983; & Peto et al., 1981*), have shown that retinol can be reasonably stable in stored samples. Experiments conducted to simulate the exposure to light, thawing and refreezing during 14 - 16 years of storage, showed retinol was also quite stable (*Kark et al., 1981*). Nierenberg et al. (1989), verified the stability of both retinol and ß-carotene in frozen plasma samples. RBP is more stable than retinol during extended storage and could be a better indicator of vitamin A status, according to Olson (1984).

2.2 BIOCHEMICAL ANALYSIS

All of the assay measurements were conducted in a "blind" manner, with identity and classification of the study population not being revealed until the analyses were complete. Additionally, each batch of samples was randomly analyzed for the biochemical parameters of concern.

In order to assess the biochemical status of vitamin A in the control subjects and patients, plasma levels of retinol, its transport proteins (RBP and prealbumin), and zinc which is required for the synthesis of RBP, were determined. Tissue zinc levels were also evaluated for patients that had breast cancer and were currently diagnosed as disease free, and benign subjects. In addition, total protein and albumin levels in the plasma were determined.

2.2.1 Total Protein Determination in the Plasma

Total plasma proteins were determined by the Biuret method (*Chan, 1983; & Nath, 1976*), using a Lambda 3 spectrophotometer (Perkin Elmer). This method is based on the principle that protein in the plasma will react with the alkaline copper solution, producing a blue colour; the intensity of which is proportional to the amount of protein present in the plasma.

The stock Biuret reagent was prepared from 45 g potassium sodium tartrate (Rochelle salt), 15 g copper sulphate and 5 g potassium iodide, dissolved in 0.2N sodium hydroxide to make 1000 mL. The working Biuret reagent consisted of 50 mL stock reagent and 1.25 g potassium iodide, dissolved in 0.2N sodium hydroxide to make 250 mL.

Aliquots of 50 μL of the thawed plasma samples were pipetted into 15 mL test tubes. Then, 2 mL of sodium sulphate (22.7%) and 5 mL of working Biuret reagent were added to each tube. After being vortexed for 10 seconds and a standing time of 10 minutes, spectrophotometric readings were taken at a wavelength of 555 nm, using the spectrophotometer. All samples were measured in duplicate and a blank was included for each run. Human protein standard solution (Behring Diagnostics), was used to obtain a standard curve for each batch of samples (example **Fig. 2-1**), and to calculate total protein concentration.

2.2.2 *Plasma Albumin and Globulin Determination*

Albumin was evaluated in the plasma samples by a modification of the Bromcresol Green method (*McPherson et al., 1972*). This is a dye-binding method which has been shown to bind specifically to albumin. The working colour reagent included 94.5 mL glycine (7.507 g glycine, dissolved in 100 mL water), and 5.5 mL 1.0 N hydrochloric acid. To this, 3 mL of 0.02 M bromcresol green concentrate (1.396 g B.C.G. in 100 mL A.R. grade absolute alcohol), was added and made up to 1 L with water. The pH was adjusted to 3.8 and the reagent stored in the refrigerator.

Aliquots of 20 μL of the thawed plasma samples were pipetted into 15 mL test tubes, at the same time as total protein determination. To this, 5 mL of the dye reagent was added and the colour change read immediately, against a reagent blank in the spectrophotometer at 635 nm, using an albumin standard (human protein standard, Behring Diagnostics). The samples were measured in duplicate and a standard curve (example **Fig. 2-2**), was used to calculate albumin concentration for each sample. Globulin concentration in the plasma was calculated as the difference between total protein and albumin concentration in each sample.

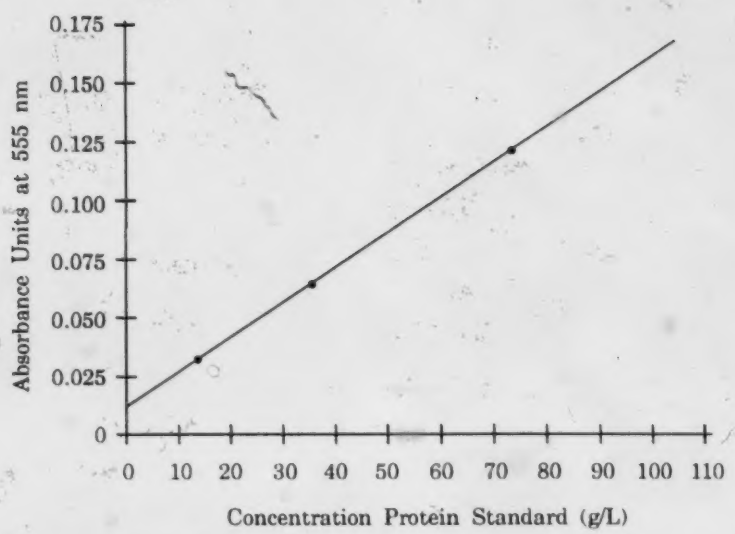

FIG 2-1. STANDARD CURVE FOR TOTAL PROTEIN

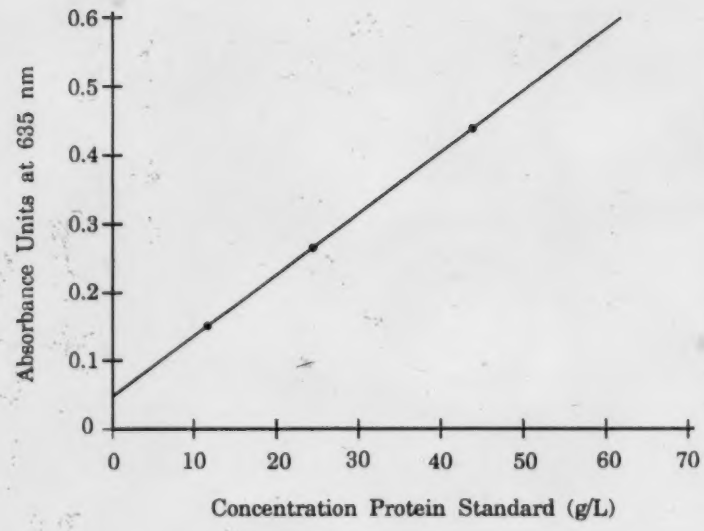

FIG 2-2. STANDARD CURVE FOR ALBUMIN

2.2.3 Retinol-Binding Protein Determination in the Plasma

Plasma RBP levels were measured quantitatively by radial immuno-diffusion assay with L.C. Partigen plates from Behring Diagnostics. This is based on the principle of an unknown amount of protein being allowed to diffuse radially from a well in a uniformly thin layer of agar, with the final area reached by the precipitate being directly proportionally to the amount of protein employed (*Mancini et al., 1965*).

Initially, the opened plates were left to stand for 5 minutes at room temperature, for evaporation of any condensation water that may have entered the wells. Aliquots of 20 μL of undiluted, thawed plasma samples were applied (using a micro syringe), to the individual wells of the plates. The plates were then allowed to diffuse radially for 72 hours at room temperature. The diameters of the resulting precipitin rings were measured using a measuring viewer (Behring Diagnostics), to an accuracy of 0.1 mm and compared with RBP standards. Protein standard plasma (Behring Diagnostics), of known protein concentration (0.102 g/L), was used to obtain a standard curve (example **Fig. 2-3**). The RBP concentration of the plasma samples was determined directly from the plot of the square of the diameter on the standard curve. Dilutions of the standard with physiological saline solution (0.9% NaCL), were of the range 1:1, 1:4 and 1:8; with 20 μL aliquots placed in the first 3 wells of the plates. The accuracy of the method was checked by pipetting a control plasma

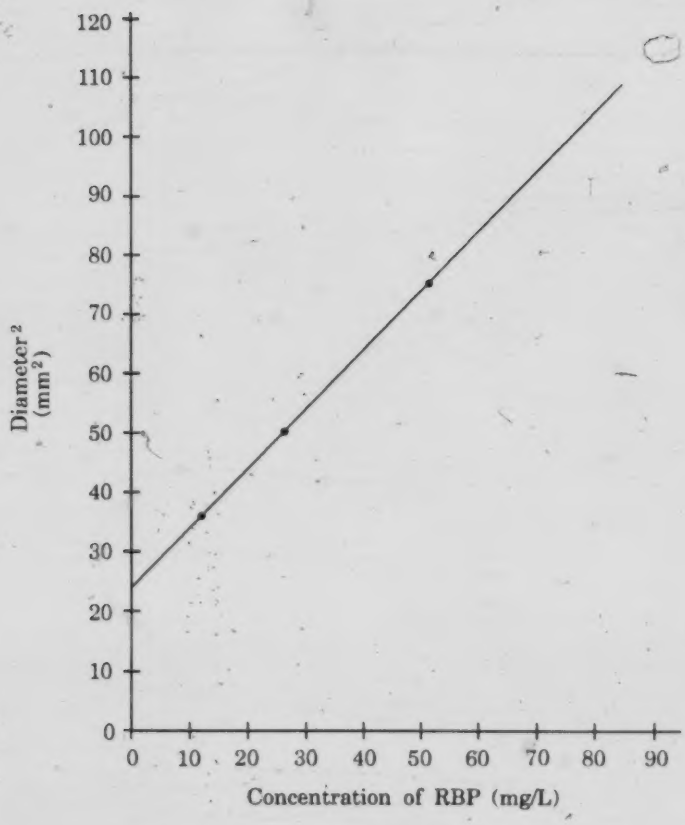

FIG 2-3.

STANDARD CURVE FOR RETINOL-BINDING PROTEIN

for Partigen (Behring Diagnostics), in one well at the same dilution as the sample being examined.

2.2.4 Plasma Prealbumin Determination

Plasma prealbumin levels of the samples were also determined by radial immunodiffusion assay with M. Partigen prealbumin plates from Behring Diagnostics. Each well of the pre-opened plate was filled with aliquots of 5μL of the undiluted thawed plasma samples. Human standard (Behring Diagnostics), with a known amount of prealbumin (0.26 g/L), was used to prepare a standard curve (**Fig. 2-4**). Samples and standards were allowed to diffuse radially for 72 hours at room temperature. The diameters of the resulting precipitin rings were measured, as indicated for retinol-binding protein.

2.2.5 Zinc Determination in the Plasma

Zinc content of the plasma was analyzed using atomic absorption spectrophotometry (AAS). The principles of atomic absorption are based on the absorption of light by free atoms of an element, at a wavelength specific to that element. The radiation which is attenuated by an amount proportional to the concentration of free atoms in the flame, enters the monochromator, which amplifies the resulting signal and presents this as a digital display (*Milner et*

al., 1984). It is recognized that zinc determination can easily be subject to contaminants and zinc can chelate to specific molecules such as the anti-coagulant EDTA (*Cousins, 1986*). To avoid contamination in the measurement of zinc, the glassware used in the experiments was rinsed in 50 g/L nitric acid solution and rinsed with deionized water (*Liska et al., 1985*). A zinc working standard at 10 ppm was prepared for each batch of samples, using 1000 μL zinc atomic absorption standard (Aldrich Chemical Co). A series of 10 standard solutions were made from the stock, at 0.1 to 1.0 ppm and at a lower range of 0.01 to 0.09 ppm, using the first series of standards. Sodium chloride solution at the same concentration of the sample solutions (0.09%) was used to dilute the standard; to compensate for the scatter effect experienced at low wave lengths. The plasma samples, which had been stored in zinc-free vacutainer tubes in the freezer at -20°C, were thawed at room temperature. Capped tubes were then vortexed for 10 seconds and 500 μL aliquots were transferred to glass culture tubes and diluted 1/10 with deionized water. The samples were capped, stored in the refrigerator and vortexed for 10 seconds before analysis.

Zinc concentrations in the series of standards and the plasma samples were read on a Philips SP9 AAS; using an air/acetylene flame; lamp current 7 mA; and wavelength 213.9 nm. Sample aspiration rate was an average 4.6 mL/min, with an integration time of 5 seconds for each of the 3 readings taken for each sample. Random checks were made of the accuracy of the standard

curve (**Fig. 2-5**), which was repeated for each run, and 10% of the plasma samples were analyzed in duplicate.

2.2.6 Tissue Zinc Determination

Zinc content was determined in 11 samples of frozen breast tissue (from 7 benign breast disease and 4 breast cancer and disease free patients). The tissue samples were weighed using an analytical balance (Sartorius model), and analyzed on a wet weight basis (*Lipman et al., 1987*). A 2-3 mg central portion of each tissue was excised with a scalpel, weighed and transferred to a 125 mL round bottom digestion flask, with cap. Concentrated sulphuric acid (4 ml H_2S0_4), was added and the samples were digested on a block heater (in a fume hood), at 110°C for 40 minutes. A 10 mL mixture of concentrated nitric acid/perchloric acid (72%)/sulphuric acid ($HNO_3/HCl0_4/H_2S0_4$; by volume 8:2:3), was added and digestion continued for a further 30 minutes, until a clear solution was produced (*Oster et al., 1989*). The cooled solution was transferred quantitatively to a 100 mL volumetric flask, diluted with deionized water and mixed.

The concentration of zinc was determined using the procedures for plasma zinc. Standards were prepared by diluting the stock standard solution with deionized water, and the water was also used as a blank solution (Perkin-Elmer, 1982).

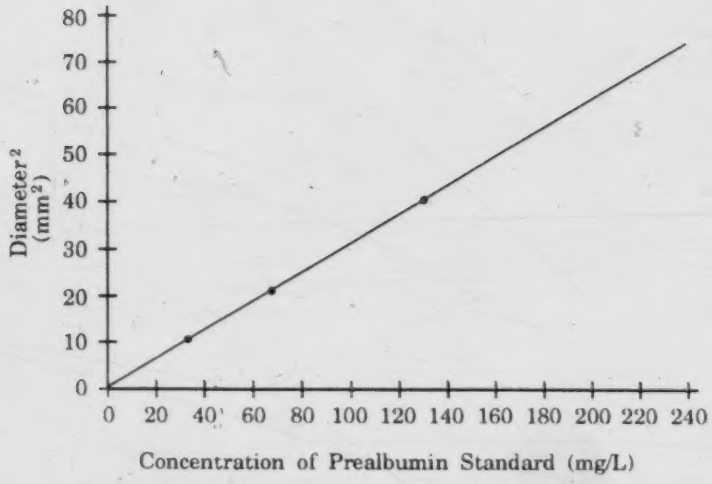

FIG 2-4. STANDARD CURVE FOR PREALBUMIN

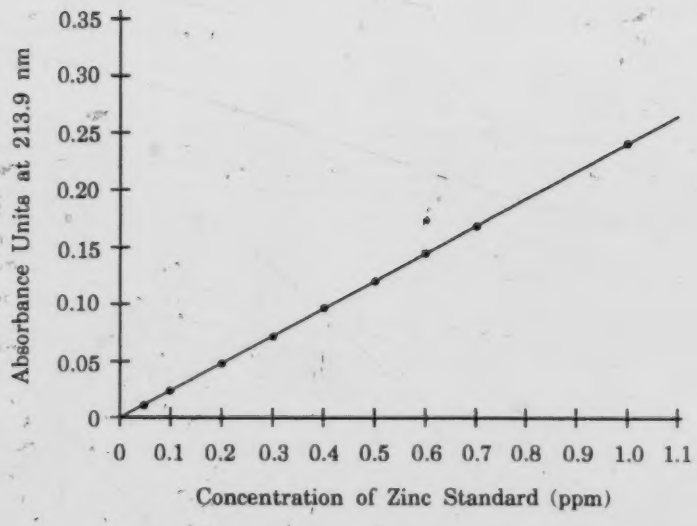

FIG 2-5. STANDARD CURVE FOR ZINC

2.2.7 Retinol Determination in the Plasma

Vitamin A content of the plasma was determined using high performance liquid chromatography (HPLC), as modified from Broich et al. (1983); Biesalski et al. (1986); Catignani et al. (1983); Driskell et al. (1982); De Ruyter et al. (1976); Nierenberg et al. (1985); and Rautahti et al. (1990). It is well recognized that this is a preferable method of analysis, compared with previously used fluorometric techniques (*Stacewicz-Sapuntzakis et al., 1987; & Schindler et al., 1985*). Modern liquid chromatography enables a rapid and non destructive analysis at room temperature and allows the separation and identification of the peaks from different vitamin A compounds, through the polarity of the mobile phase. In a reversed phase system, retention is not due to favourable interaction with the stationary phase, but to the effect of the mobile phase forcing the compound into the hydro-carbon stationary phase (*De Leeheer et al., 1985*).

The HPLC equipment used consisted of a Perkin-Elmer chromatographic pump (series 3B liquid chromatograph); a Varian wavelength detector; and a Perkin Elmer chart recorder for reading the chromatographs. A reversed phase (Partisal SODS-3), C18 column was used with an isocratic solvent system, methanol: water (85:15). All solvents used were HPLC grade and the mobile phase was de-gassed before each run. The concentrated standards were covered in aluminum foil and stored in the freezer (at -20°C). All procedures

were also performed in dim light, since vitamin A is sensitive to ultraviolet light (*Hunter, 1990*).

For the standard, crystalline all-trans retinol (Aldrich Chemical Co.) was dissolved in acetonitrile and dilutions were made in a range, similar to the amounts of compound present in the injected test samples. The standard solutions were: 0.5, 1.0, 2.0 and 2.5 μ/mL. Standard concentrations were made repeatedly on a daily basis before running each batch of samples. From this, standard curves (example, **Fig. 2-6**) were generated, comparing quantity of compound versus integrated peak areas.

For the extraction of retinol from the plasma samples aliquots of 100 μL of thawed plasma (previously stored at -20°C and brought to room temperature), were pipetted into microcentrifuge tubes. The internal standard, 100 μL of crystalline all-trans retinol acetate (Sigma Chemical Co.), dissolved in acetonitrile at 4.0 μg/mL concentration, was added and vortexed for 15 seconds. An additional 250 μL of butanol:ethyl acetate (1:1) was added to each tube, vortexed again for 60 seconds and 150 μL of potassium phosphate (K_2HP0_4), was added to de-proteinize the plasma. This was vortexed for a further 30 seconds and centrifuged at 9000 rpm for 1 minute, to separate the phases. The organic upper layer was carefully removed using a Pasteur pipette, transferred to another microcentrifuge tube and centrifuged again at 9000 rpm for 1 minute. The capped tubes were covered with aluminium foil and placed

on ice to prevent evaporation and light absorption while waiting for HPLC analysis.

From this extract, 50 μL aliquots were injected for routine chromatography, performed at ambient temperatures. The wave length was set at 325 nm for retinol absorption maxima and sensitivity was set at 0.08A full-scale. Retinol content was calculated from a standard curve of the ratio of its peak height to that of retinol acetate, using a computer SAS program for regression analysis of the data. A linear relationship between peak height ratios (peak height of retinol: peak height of retinol acetate), and concentration (concentration of retinol: retinol acetate) was found. A typical chromatogram of plasma extract, containing retinol acetate as an internal standard, is shown in **Fig. 2-7**. The total retention time for the assay with a flow rate of 1.25 mL/min was on average 6 minutes.

2.3 STATISTICAL ANALYSIS

All the statistical analyses were carried out using SAS computerized procedures. The three categories of breast disease and the healthy control group were compared with respect to their individual parameters, using a one-way analysis of variance. Differences between group means were analyzed for statistical significance by the Student's t-test and Duncan's New Multiple Range Test. Pearson's Correlation Coefficients, relating retinol with its carrier

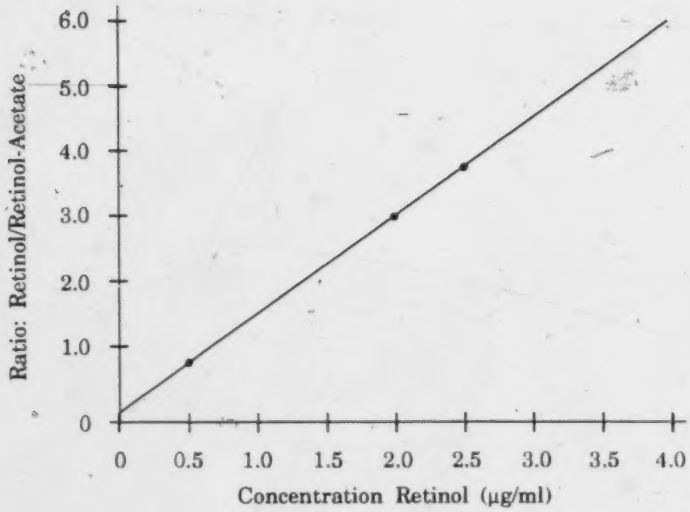

FIG 2-6. STANDARD CURVE FOR RETINOL

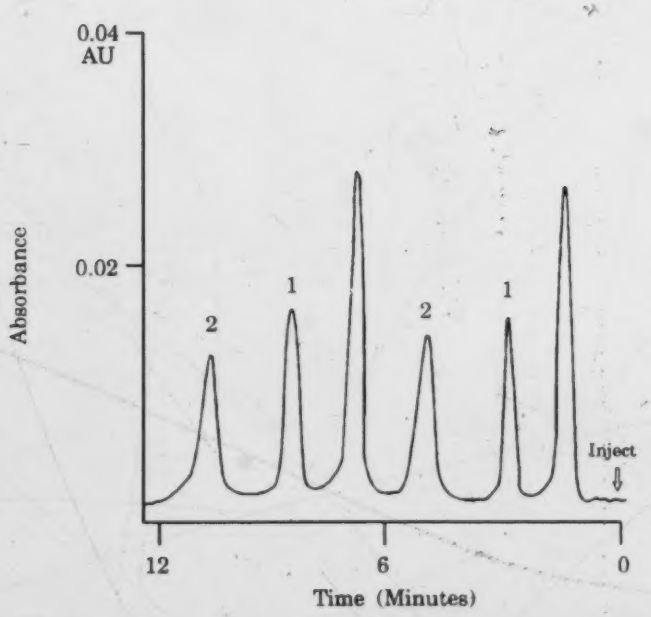

FIG 2-7. HPLC CHROMATOGRAM OF INJECTION OF PLASMA EXTRACTS

Peaks: 1 = retinol; 2 = retinol acetate

proteins, were determined. Canonical discriminant analysis (from both SAS and SPSS), was used to evaluate the relationship of the vitamin A parameters with their designated subject or patient group.

3. RESULTS

3.1 BIOCHEMICAL STATUS OF VITAMIN A AND BREAST DISEASES

Table 3-1 shows the plasma levels of retinol and its carrier proteins in the study population. It is evident that the subjects with benign breast disease and the patients with either metastatic breast cancer or post-operative disease free status, had lower levels of plasma retinol compared to the healthy control subjects. According to the National Health and Nutrition Examination Surveys (NHANES), the individuals with plasma retinol levels below 0.7-1.0 μmol/L (20-30 μg/dL) would be considered to be associated with vitamin A deficiency (*Pilch, 1987*). None of the subjects/patients in the study population had plasma retinol levels below this acceptable range.

Retinol-binding protein levels were considerably less ($P < 0.01$) in all patients, compared with the healthy controls. Specifically, from the total study population, one of the individuals diagnosed with metastatic breast cancer had a RBP plasma level below the normal range of 26-76 mg/L (*Gibson, 1990*), and two individuals from the same group were on the verge of subnormal values. It is noteworthy that all three patients are now deceased. It is of further interest, that 76% of this group (and 44% of the post-operative disease free breast cancer patients), had plasma RBP values below the normal average of 45 mg/L, as reported by Rask et al. (1980), compared with only 16% of the individuals from the control group. Levels of plasma prealbumin were lowest

TABLE 3-1

Plasma levels of retinol and its carrier proteins in the study population[1,2]

BIOCHEMICAL PARAMETERS	CONTROLS (n = 25)	BENIGN BREAST DISEASE (n = 28)	POST-OPERATIVE DISEASE FREE BREAST CANCER (n = 25)	METASTATIC BREAST CANCER (n = 17)
RETINOL (μmol/L)	3.4 ± 0.3[a] n = 24	2.5 ± 0.2[b] n = 24	2.4 ± 0.2[b] n = 23	2.8 ± 0.4[ab] n = 17
RETINOL-BINDING PROTEIN (mg/L)	53.3 ± 2.4[a*]	42.9 ± 1.2[b*]	45.3 ± 1.4[b*]	40.5 ± 3.1[b*]
PREALBUMIN (mg/L)	215.1 ± 9.8[ab]	228.4 ± 9.0[a]	208.1 ± 9.8[ab]	183.8 ± 14.8[b]

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated.
[2]In each row, values not sharing a common superscript letter are significantly different at $P < 0.05$, * $P < 0.01$.

in the breast cancer patients (**Fig. 3-1**), compared to the controls, although not significantly different. From the study population, one patient with metastatic breast cancer had a plasma prealbumin level below the normal range of 100-240 mg/L, as reported by Gibson (1990).

The molecular ratio of retinol and its carrier proteins (**Table 3-2**), confirmed that retinol exists as a trimolecular complex with RBP and prealbumin, for transportation within the plasma. All values approached a ratio of 1:1, with a ratio lower than 1.0 indicating a greater proportion of free retinol is not completely combined with the apo-RBP. In the plasma, most of the RBP normally circulates as the retinol-RBP complex (holo-RBP), (*Smith et al., 1979*). The correlations between assay measurements of retinol and its carrier proteins (**Table 3-3**), indicated a positive significant relationship between retinol and RBP in all groups, apart from the benign breast disease subjects. Retinol positively and significantly correlated with prealbumin in the controls and patients with metastatic breast cancer. A statistically significant positive relationship was also seen between the RBP and prealbumin in all four groups.

Canonical discriminant analysis was used to confirm the membership of an observed individual to one of a given set of populations, to which she can possibly belong (*Hodgson, 1987; & Kergoat et al., 1987*). From the statistical analysis, 52.9% of the breast cancer patients; 26.1% of the post-operative disease free breast cancer patients, 41.7% of the benign breast disease and

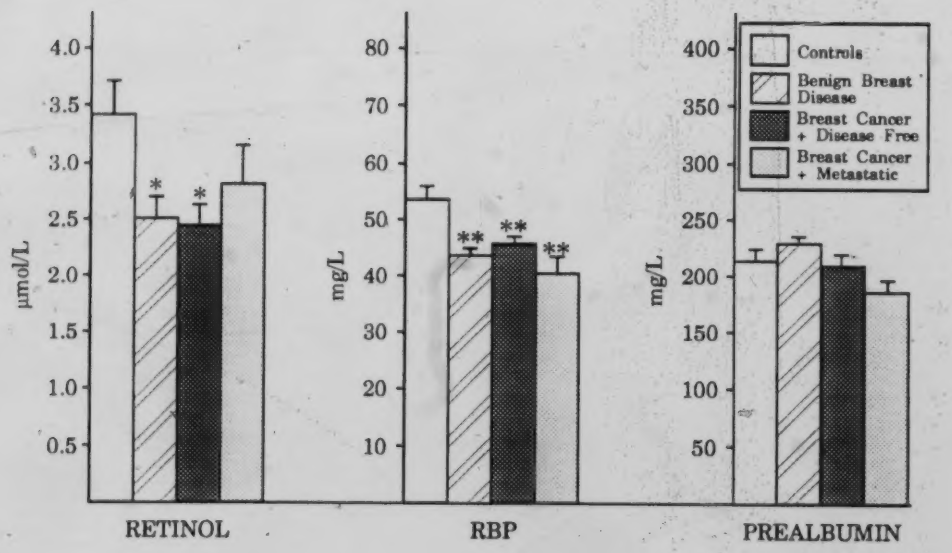

FIG 3-1. Comparison of plasma levels of retinol, retinol-binding protein and prealbumin in all 4 groups of the study population. Bars represent means ± SEM.

* Significantly different from controls as determined by Duncan's New Multiple Range Test (*P<0.05, **P<0.01).

TABLE 3-2

Molecular ratio of the carrier proteins with retinol in the study population[1,2,3]

MOLECULAR RATIOS	CONTROLS (n = 24)	BENIGN BREAST DISEASE (n = 24)	POST-OPERATIVE DISEASE FREE BREAST CANCER (n = 23)	METASTATIC BREAST CANCER (n = 17)
RETINOL-BINDING PROTEIN/ RETINOL	0.86 ± 0.07[a]	0.88 ± 0.04[a]	1.02 ± 0.09[a]	0.82 ± 0.08[a]
PREALBUMIN/ RETINOL	1.38 ± 0.15[a]	1.81 ± 0.12[a]	1.80 ± 0.18[a]	1.40 ± 0.13[a]

[1]Values are means ± SEM for the number of subjects shown in parenthesis.

[2]In each row, values not sharing a common superscript letter are significantly different at $P < 0.05$.

[3]Values are calculated according to the molecular weight of retinol (286.5), and its carrier proteins (RBP 20.1 kDa and Prealbumin 54.9 kDa), e.g. individual values of RBP (mg/L)/20.1 gives µmol/L. Ratios calculated using SAS.

TABLE 3-3

Correlations between assay measurements of retinol and its carrier proteins in the study population

GROUPS	NUMBER OF SUBJECTS	PEARSON'S CORRELATION COEFFICIENT (r)	P-VALUE
CONTROLS			
RETINOL WITH RBP	24	0.77	0.0001
RETINOL WITH PREALBUMIN	24	0.52	0.0092
RBP WITH PREALBUMIN	25	0.58	0.0024
BENIGN BREAST DISEASE			
RETINOL WITH RBP	24	0.38	0.065
RETINOL WITH PREALBUMIN	24	0.14	0.507
RBP WITH PREALBUMIN	28	0.39	0.041
POST-OPERATIVE DISEASE FREE BREAST CANCER			
RETINOL WITH RBP	23	0.42	0.047
RETINOL WITH PREALBUMIN	23	0.28	0.189
RBP WITH PREALBUMIN	25	0.40	0.049
METASTATIC BREAST CANCER			
RETINOL WITH RBP	17	0.75	0.0005
RETINOL WITH PREALBUMIN	17	0.73	0.0008
RBP WITH PREALBUMIN	17	0.76	0.0004
TOTAL SUBJECTS			
RETINOL WITH RBP	88	0.68	0.0001
RETINOL WITH PREALBUMIN	88	0.41	0.0001
RBP WITH PREALBUMIN	95	0.50	0.0001

54.2% of the controls were correctly classified. However, only 43.2% of the total "grouped" cases were correctly classified according to the discriminant analysis.

3.2 STAGES OF BREAST CANCER AND THE STATUS OF VITAMIN A

Table 3-4 shows the effect of time intervals between surgery and blood sample collection on the plasma levels of retinol and its carrier proteins, of the post mastectomy patients. The time intervals ranged from <2 months to >24 months. The type of surgery involved are given in **Table 2-1**. The type of surgery or the time lapsed between the surgery and blood collection did not appear to have any significant effect on the plasma levels of retinol, RBP and prealbumin.

The results from **Table 3-5** highlight the effect of the progression of the disease in the advanced cancer patients, on the plasma levels of retinol and its carrier proteins. While not significantly different, possibly due to the small sample size, it is noticeable that at clinical stage IV (where there is metastases present), the plasma values for retinol, RBP and prealbumin were all less than at clinical stage III (for example RBP, 37.0 ± 3.2 mg/L versus 43.7 ± 5.0 mg/L), (mean ± SEM). Since the beginning of this study, seven of the reported metastatic breast cancer patients have died. **Table 3-6** illustrates that the deceased patients had lower mean plasma levels of retinol, RBP and pre-

TABLE 3-4

Effect of time interval between surgery and blood sample collection on plasma levels of retinol and its carrier proteins in the post-operative breast cancer patients[1,2]

BIOCHEMICAL PARAMETERS	<2* (n=14)	2-6 (n = 3)	6-12 (n = 2)	12-24 (n=5)	>24 (n = 18)
RETINOL (μmol/L)	2.45 ± 0.32[a]	4.12 ± 0.76[a]	2.52 ± 1.20[a]	2.23 ± 0.48[a] n = 4	2.44 ± 0.36[a] n = 17
RETINOL-BINDING PROTEIN (mg/L)	46.52 ± 1.93[a]	40.07 ± 7.87[a]	38.45 ± 2.55[a]	43.76 ± 4.13[a]	41.89 ± 2.74[a]
PREALBUMIN (mg/L)	218.03 ± 12.54[a]	179.33 ± 39.06[a]	181.05 ± 11.95[a]	195.84 ± 12.36[a]	188.7 ± 15.54[a]

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated.
[2]In each row, values not sharing a common superscript letter are significantly different at $P < 0.05$.
*Number of months.

TABLE 3-5

Effect of clinical staging of the disease on plasma levels of retinol and its carrier proteins in advanced breast cancer patients[1]

BIOCHEMICAL PARAMETERS	CLINICAL STAGE III (n = 9)	CLINICAL STAGE IV (n = 8)	P-VALUE
RETINOL (μmol/L)	3.3 ± 0.7	2.2 ± 0.3	0.181
RETINOL-BINDING PROTEIN (mg/L)	43.7 ± 5.0	37.0 ± 3.2	0.298
PREALBUMIN (mg/L)	191.6 ± 25.0	175.1 ± 15.6	0.595

[1]Values are means ± SEM for the number of subjects shown in parenthesis.
[2]Probability levels, from the Student's t-test show no significant difference between means.

TABLE 3-6

Plasma levels of retinol and its carrier proteins in the metastatic breast cancer patients and those subsequently who died of cancer[1]

BIOCHEMICAL PARAMETERS	METASTATIC BREAST CANCER (n = 10)	METASTATIC BREAST CANCER DECEASED (n = 7)	P-VALUE
RETINOL (μmol/L)	2.96 ± 0.6	2.48 ± 0.4	0.574
RETINOL-BINDING PROTEIN (mg/L)	43.9 ± 4.5	35.7 ± 3.4	0.200
PREALBUMIN (mg/L)	204.5 ± 20.2	154.3 ± 17.6	0.097

[1]Values are means ± SEM for the number of subjects shown in parenthesis.
[2]Probability levels, from the Student's t-test show no significant difference between means.

albumin (compared to the patients who are still alive). However, the differences between the means were not found to be statistically significant.

3.3. BIOCHEMICAL STATUS OF PROTEIN AND ZINC

Since general protein status in an important factor affecting vitamin A status (*Gibson, 1990*), the protein status of the study population was assessed by the evaluation of total protein and albumin levels in the plasma. **Table 3-7** indicates that there were no statistically significant differences in plasma total protein between the patients with breast diseases and the healthy control subjects. There was, however, a consistent trend in that the patients with malignant and benign breast disease had lower plasma protein levels than the healthy subjects. Like the total protein, plasma albumin values showed no significant differences between the four study groups, and the values tended to be lower for those patients with all breast diseases than the healthy subjects. The albumin/globulin ratio was also found to be within the normal acceptable range (*Gibson, 1990*), indicating normal protein status in all groups of the study population.

Zinc is another factor which is involved in the synthesis of RBP, and therefore affects the metabolic availability of retinol from the liver (*Bates et al., 1981*). As shown in **Table 3-8**, the plasma zinc values for all of the study

TABLE 3-7

Plasma protein levels in the study population[1,2]

BIOCHEMICAL PARAMETERS	CONTROLS (n = 25)	BENIGN BREAST DISEASE (n = 28)	POST-OPERATIVE DISEASE FREE BREAST CANCER (n = 25)	METASTATIC BREAST CANCER (n = 17)
TOTAL PROTEIN (g/L)	79.7 ± 2.1[a]	75.3 ± 1.5[a]	75.1 ± 1.9[a]	75.1 ± 2.2[a]
ALBUMIN (g/L)	46.3 ± 1.2[a]	46.0 ± 0.8[a]	45.8 ± 0.9[a]	45.8 ± 1.4[a]
GLOBULIN (g/L)	33.4 ± 1.9[a]	29.3 ± 1.7[a]	29.3 ± 2.1[a]	29.3 ± 1.7[a]
ALBUMIN/GLOBULIN RATIO	1.58 ± 0.17[a]	1.80 ± 0.17[a]	1.77 ± 0.14[a]	1.65 ± 0.13[a]

[1]Values are means ± SEM for the number of subjects shown in parenthesis.

[2]In each row, values not sharing a common superscript letter are significantly different at $P < 0.05$.

TABLE 3-8

Zinc concentrations in plasma and breast tissue samples of the study population[1,2,3]

BIOCHEMICAL PARAMETERS	CONTROLS (n = 25)	BENIGN BREAST DISEASE (n = 28)	POST-OPERATIVE DISEASE FREE BREAST CANCER (n = 25)	METASTATIC BREAST CANCER (n = 17)
PLASMA ZINC (μmol/L)	23.3 ± 3.7[b]	39.1 ± 5.8[a]	44.4 ± 6.3[a]	19.3 ± 4.4[b]
TISSUE ZINC (μmol/g)	N/A	5.3 ± 0.4 n = 7	5.1 ± 0.7 n = 4	N/A

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated (N/A = subjects not available).
[2]In the row for plasma zinc, values not sharing a common superscript letter are significantly different at $P < 0.05$.
[3]No significant difference between means for tissue zinc (Student's t-test).

population fell within an acceptable amount, which is not less than 10.71 μmol/L (70 μg/dL), (*Gibson, 1990*). However, it was of interest that the patients with benign breast disease and post-operative disease free status had significantly higher plasma zinc concentrations, than in either healthy subjects or the patients with metastatic breast cancer.

Biopsy breast tissue samples were obtained from 7 benign breast disease and 4 post-operative disease free breast cancer patients. There was no significant difference in the amount of zinc found in the breast tissue between the two groups of patients. The mean value 5.2 μmol/g (33.8 μg/g wet weight), can be related to the approximate zinc content of the major organs and tissues in the normal adult (*Jackson, 1989*), (**Appendix #11**). The Pearson's correlation coefficient was used to evaluate the possibility of a relationship between plasma and tissue zinc levels within these 11 samples; but no statistically significant correlations were found.

3.4 RELATIONSHIPS BETWEEN LIVER FUNCTION, CHOLESTEROL AND VITAMIN A STATUS

Liver function tests in all breast cancer patients were carried out by the hospital laboratories. These tests included alkaline phosphatase, lactate dehydrogenase (LDH), serum glutamic oxaloacetic transaminase (SGOT) and total bilirubin. Normal values are given in **Appendix #7B**. Abnormally high

values were recorded in 12 of the 42 breast cancer patients. Presumably this could indicate some metastatic spread of the cancer to the liver, which in turn could affect the synthesis of RBP by the hepatocytes. **Table 3-9** shows that the values for retinol, RBP and prealbumin were all reduced in the patients with abnormal liver function, compared to those with normal liver functions. The differences between the two groups for all 3 parameters, were not statistically significant.

Five of the 42 breast cancer patients were found to have abnormally high blood cholesterol levels (greater than 6.5 mmol/L). It was of interest that the patients with hypercholesterolemia also had non-significantly lower levels of retinol, RBP and prealbumin in the plasma, when compared with the normo-cholesterolemic patients (**Table 3-10**).

The Body Mass Index of the study population and its effect on vitamin A status is presented in **Table 3-11**. The BMI is divided into three representative groups: normal weight, overweight and obese, as specified by Wurtman et al., 1987. The majority (53%) of the total study population can be classified as "normal" weight, with 27% and 19% classified as overweight and obese, respectively. There were no significant differences with retinol and RBP levels between each of the groups, but prealbumin levels were significantly lower in the obese group.

TABLE 3-9

Effect of liver function tests on plasma levels of retinol and its carrier proteins in the breast cancer patients[1]

BIOCHEMICAL PARAMETERS	NORMAL LIVER FUNCTION (n = 30)	ABNORMAL LIVER FUNCTION (n = 12)	P-VALUE
RETINOL (μmol/L)	2.61 ± 0.23 n = 28	2.42 ± 0.48	0.683
RETINOL-BINDING PROTEIN (mg/L)	43.9 ± 1.4	42.0 ± 4.1	0.587
PREALBUMIN (mg/L)	204.8 ± 9.2	181.9 ± 18.5	0.227

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated.
[2]Probability levels, from the Student's t-test show no significant difference between means.

TABLE 3-10

Effect of blood cholesterol levels on plasma levels of retinol and its carrier proteins in the post-operative breast cancer patients[1]

BIOCHEMICAL PARAMETERS	NORMAL BLOOD CHOLESTEROL (n = 37)	ABNORMAL BLOOD CHOLESTEROL (n = 5)	P-VALUE
RETINOL (μmol/L)	2.61 ± 0.23 n = 35	2.12 ± 0.42	0.450
RETINOL-BINDING PROTEIN (mg/L)	43.6 ± 1.7	41.6 ± 2.3	0.671
PREALBUMIN (mg/L)	200.4 ± 9.3	182.4 ± 18.5	0.498

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated.
[2]Probability levels, from the Student's t-test show no significant difference between means.

TABLE 3-11

Effect of body mass index (BMI) on plasma levels of retinol and its carrier proteins in the study population[1,2]

	CONTROLS			BENIGN BREAST DISEASE			POST-OPERATIVE DISEASE FREE BREAST CANCER			METASTATIC BREAST CANCER			TOTAL SUBJECTS		
	<25 (n=11)	25-30 (n=10)	>30 (n=4)	<25 (n=20)	25-30 (n=4)	>30 (n=3)	<25 (n=7)	25-30 (n=6)	>30 (n=7)	<25 (n=7)	25-30 (n=6)	>30 (n=4)	<25 (n=50)	25-30 (n=26)	>30 (n=18)
RETINOL (μmol/L)	3.3 ± 0.4	3.9 ± 0.8	2.8 ± 0.1	2.6 ± 0.2	2.1 ± 0.3	2.4	2.6 ± 0.4	2.3 ±0.1	2.1 ±0.2	2.9 ± 0.8	3.1 ±0.6	2.0 ± 0.3	2.8[a] ± 0.2	3.0[a] ± 0.3	2.3[a] ± 0.2
		n=9		n = 18		n=1	n=11		n=6				n=47	n=25	n=15
RETINOL-BINDING PROTEIN (mg/L)	49.6 ± 2.0	56.7 ± 5.3	27.3 ± 5.2	42.6 ± 5.1	41.5 + 1.9	45.7 ± 1.9	44.6 ± 2.1	42.7 ± 2.1	48.7 ± 2.5	43.8 ± 6.5	40.4 ± 4.3	34.3 ± 1.4	44.8[a] ± 1.3	47.5[a] ± 2.7	46.4[a] ± 2.2
PREALBUMIN (mg/L)	217.2 ± 9.4	230.2 ± 20.0	171.0 ± 13.2	229.0 ± 11.5	220.8 ± 24.5	217.0 ± 1.8	218.2 ± 16.0	211.4 ± 20.7	188.2 ± 13.0	180.1 ± 30.9	207.4 ± 20.2	154.8 ± 8.7	217.0[a] ± 7.7	219.2[a] ± 10.4	181.7[b] ± 7.5

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated.

[2]In each row, values for the total subjects not sharing a common superscript letter are significantly different at $P < 0.05$.

* BMI < 25 kg/m^2 = "normal" weight; BMI 25-30 kg/m^2 = overweight; BMI > 30 kg/m^2 = obese (*Wurtman et al., 1987*).

3.5 ADDITIONAL FACTORS AFFECTING RETINOL AND ITS CARRIER PROTEINS

The influence of adjuvant treatment on the vitamin A status of the breast cancer patients is shown in **Table 3-12**. Previously, it has been reported that plasma vitamin A levels of cancer patients are decreased during the period of radiotherapy (*Schreurs et al., 1985*). In this present study, the breast cancer patients receiving any type of adjuvant treatment, including radiotherapy were also found to be associated with decreased plasma concentrations of not only retinol, but also its carrier protein, prealbumin. However, the differences were not found to be statistically significant, as a result of the small sample sizes obtained for the different adjuvant treatments.

Table 3-13 shows the effect of family history on the biochemical status of vitamin A. This is important, since genetic influence has been shown to be an identifiable risk factor (*Osborne, 1991*), on the plasma levels of retinol and its carrier proteins. There appeared to be no significant difference in retinol status between the patients with benign breast disease and the breast cancer. However, it is noteworthy that age (**Table 3-14**) did have an effect on plasma levels of RBP, in that the patients and the healthy subjects beyond the age of 55 had significantly higher levels of this protein ($P < 0.05$). Plasma concentrations of retinol and prealbumin remained unaffected by the difference retinol and prealbumin remained unaffected by the difference in age. Post-

TABLE 3-12

Effect of adjuvant treatment on plasma levels of retinol and its carrier proteins in the post-operative breast cancer patients[1]

BIOCHEMICAL PARAMETERS	NO TREATMENT (n = 15)	CHEMOTHERAPY (n = 2)	RADIOTHERAPY (n = 9)	HORMONE THERAPY (n = 6)	CHEMO-RADIO-THERAPY (n = 5)	CHEMO-HORMONE-THERAPY (n = 1)	RADIO-HORMONE-THERAPY (n = 1)	CHEMO-RADIO-HORMONE-THERAPY (n = 3)
RETINOL (μmol/L)	2.55 ± 0.42 n = 14	1.74 ± 0.05	2.43 ± 0.25 n = 8	2.11 ± 0.34	2.40 ± 0.24	3.83	2.45	4.16 ± 1.75
RETINOL-BINDING PROTEIN (mg/L)	45.4 ± 1.9	34.0 ± 1.0	45.5 ± 2.8	42.5 ± 1.6	36.8 ± 4.1	27.6	49.8	48.9 ± 15.0
PREALBUMIN (mg/L)	216.5 ± 7.9	144.8 ± 10.7	198.3 ± 19.2	187.1 ± 33.8	190.9 ± 22.0	134.1	169.1	208.6 ± 61.7

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless othewise stated.

TABLE 3-13

Effect of family history on plasma levels of retinol and its carrier proteins in benign breast disease and post-operative breast cancer patients[1,2]

BIOCHEMICAL PARAMETERS	BENIGN BREAST DISEASE		POST-OPERATIVE DISEASE FREE BREAST CANCER		METASTATIC BREAST CANCER		TOTAL SUBJECTS	
	FH* (n = 2)	NFH (n = 26)	FH (n = 3)	NFH (n = 22)	FH (n = 2)	NFH (n = 15)	FH (n = 7)	NFH (n = 63)
RETINOL (μmol/L)	3.2 ± 0.5	2.4 ± 0.2	3.6 ± 1.3	2.2 ± 0.2 n = 20	1.7 ± 0.2	2.9 ± 0.5	2.9 ± 0.6	2.5 ± 0.2 n = 57
RETINOL-BINDING PROTEIN (mg/L)	55.3 ± 3.1	41.9 ± 1.0	43.1 ± 7.7	45.6 ± 1.3	36.7 ± 6.5	41.1 ± 3.4	44.7 ± 4.4	43.0 ± 1.0
PREALBUMIN (mg/L)	283.0 ± 53.7	224.2 ± 8.7	192.3 ± 41.2	210.3 ± 10.1	175.1 ± 28.1	185.0 ± 16.6	213.3 ± 27.4	210.0 ± 6.6

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated.
[2]No significant difference between means for total subjects (Student's t-test).
*FH = family history, NFH = no family history.

TABLE 3-14

Effect of age group on plasma levels of retinol and its carrier proteins in the study population[1]

BIOCHEMICAL PARAMETERS	CONTROLS		BENIGN BREAST DISEASE		POST- OPERATIVE DISEASE FREE BREAST CANCER		METASTATIC BREAST CANCER		TOTAL SUBJECTS	
	< 55 y* (n = ϕ)	> 55 y (n = 25)	< 55 y (n = 23)	> 55 y (n = 5)	< 55 y (n = 5)	> 55 y (n = 20)	< 55 y (n = 3)	> 55 y (n = 14)	< 55 y (n = 31)	> 55 y (n = 64)
RETINOL (μmol/L)		3.43 ± 0.34 n = 24	2.29 ± 0.14 n = 20	3.37 ± 0.54 n = 4	2.21 ± 0.28 n = 4	2.43 ± 0.26 n = 19	4.31 ± 1.65	2.42 ± 0.32	2.7 ± 1.6 n= 27	2.9 ± 1.8 n = 61
RETINOL-BINDING PROTEIN (mg/L)		53.3 ± 2.4	41.9 ± 1.2	47.4 ± 3.6	41.6 ± 2.6	46.2 ± 1.5	50.2 ± 14.2	38.5 ± 2.4	42.7 ± 1.5†	47.4 ± 1.4
PREALBUMIN (mg/L)		215.0 ± 9.8	225.4 ± 9.8	242.3 ± 2.4	213.9 ± 24.5	206.7 ± 11.0	249.0 ± 47.1	169.9 ± 13.1	225.8 ± 9.0	204.7 ± 6.6

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated.
†Significantly different from age > 55 years for total subjects, $P < 0.05$ (Student's t-test).
* < 55 y = less than age 55 years, > 55 y = greater than age 55 years.

menopausal women, including both healthy subjects and patients also had significantly higher RBP, lower prealbumin and unchanged retinol levels in plasma, when compared with the pre-menopausal women (**Table 3-15**).

TABLE 3-15

Effect of menopausal state on plasma levels of retinol and its carrier proteins in the study population[1]

BIOCHEMICAL PARAMETERS	CONTROLS		BENIGN BREAST DISEASE		POST-OPERATIVE DISEASE FREE BREAST CANCER		METASTATIC BREAST CANCER		TOTAL SUBJECTS	
	pre-m* (n = ϕ)	post-m (n = 25)	pre-m (n = 18)	post-m (n = 10)	pre-m (n = 6)	post-m (n = 19)	pre-m (n = 3)	post-m (n = 14)	pre-m (n = 27)	post-m (n = 68)
RETINOL (μmol/L)		3.43 ± 0.3 n = 24	2.3 ± 0.2 n = 20	2.7 ± 0.3 n = 9	2.2 ± 0.2 n = 5	2.43 ± 0.3 n = 18	3.9 ± 1.9	2.5 ± 0.3	2.5 ± 0.3 n= 23	2.9 ± 0.2 n = 65
RETINOL-BINDING PROTEIN (mg/L)		53.3 ± 2.4	41.8 ± 1.4	44.9 ± 2.2	39.6 ± 21.2	4.7 ± 1.5	45.7 ± 1.7	39.4 ± 2.1	41.7 ± 1.9 †	47.5 ± 1.3
PREALBUMIN (mg/L)		215.0 ± 9.8	235.1 ± 10.6	216.5 ± 16.4	208.3 ± 21.7	208.1 ± 1.1	232.0 ± 62.1	173.5 ± 12.5	228.8 ± 10.4 ‡	204.8 ± 6.1

[1]Values are means ± SEM for the number of subjects shown in parenthesis, unless otherwise stated.

† Significantly different from post-m subjects for total subjects, $P < 0.01$ (Student's t-test).

‡ Significantly different from post-m subjects for total subjects, $P < 0.05$ (Student's t-test).

* pre-m = pre-menopausal, post-m = post-menopausal.

4. *DISCUSSION*

Many case control studies have been reported in relation to vitamin A status and cancer. There exists, however, controversial evidence as many studies have indicated a positive correlation between low plasma levels of retinol and cancer risk (*Basu et al., 1988; Knekt et al., 1990; & Potischman et al., 1990*), whilst others have shown a negative relationship (*Coates et al., 1988; Russell et al., 1988; & Willet et al., 1984*). In most of these studies seemingly healthy subjects were used as controls; vitamin A levels were determined in plasma samples stored for many years and usually collected for secondary objectives; and only plasma values for vitamin A were used as a sole index for the vitamin status.

Vitamin A is stored in the liver. The plasma level is regulated principally by release from hepatic stores and is relatively independent of short-term fluctuations in dietary intake of vitamin A. Therefore, except in cases of extreme deficiency or excess, levels of vitamin A in the plasma are not a good indicator of the vitamin status (*Olson, 1984*). Additionally, plasma vitamin A may be affected by some disease states (*Underwood, 1974 & 1984*); it is depressed in acute infections and febrile states, and can be elevated in renal disease.

The present study was conducted to investigate the biochemical status of vitamin A in breast cancer patients and to determine how this compares with

that of not only healthy subjects, but also of patients with benign breast disease. The use of the latter group as controls was thought to be essential, since the patients had benign breast disease at the same site as in the patients with breast cancer. The present study was further validated by the fact that the plasma samples were collected for the specific purpose of this study. They were obtained from rural hospitals, where surgeons had complete supervision over their patients. The plasma samples were carefully stored and not re-used once thawed.

In order to determine the biochemical status of vitamin A, the present study investigated not only its level in the plasma, but also the levels of RBP and prealbumin. Since plasma retinol levels alone are not a determinant factor for vitamin A status, it is important that plasma retinol concentration is measured along with its carrier proteins. This will suggest the status of liver storage and its metabolic availability. The plasma levels of vitamin A were found to be significantly lower in patients with breast cancer than in age and sex-matched healthy subjects. These findings are in agreement with the results obtained by others (*Atukorala et al., 1979; Husami et al., 1986; & Wald et al., 1980*). However, the differences disappeared when the breast cancer patients whether metastatic or disease free, were compared with the patients with benign breast disease. These results indicate that the depressed level of vitamin A was not a specific effect of the breast cancer.

Very few studies have determined the plasma levels of RBP and prealbumin in breast cancer patients. The present study revealed that in parallel with retinol, plasma values for its carrier protein, RBP were significantly lower in the patients with breast cancer than in healthy control subjects. A significant decrease in plasma RBP level has also been reported in pancreatic patients, compared with controls (*Fabris et al., 1984*). However, like vitamin A there was no significant difference in RBP levels between breast cancer and benign breast disease patients. These findings are similar to the results obtained in a recent study, involving patients with colorectal cancer (*Basu et al., 1991*). Not only serum levels of vitamin A, but also its transport proteins including RBP and prealbumin, were significantly lower in patients with colorectal cancer than in age and sex-matched controls. However, no significant differences in these biochemical parameters were apparent when the cancer patients were compared with a group of patients with benign colorectal disease.

According to the National Health and Nutrition Examination Surveys (*Pilch, 1988*), the mean plasma retinol values were not subnormally low in the study. However, from a comparison of the normal average plasma values for RBP (*Rask et al., 1990*), 76% of the metastatic breast cancer patients and 44% of the post-operative disease free breast cancer patients had less than the average level, compared to 16% of the healthy controls. The plasma RBP also

appears to be more severely affected when the breast disease has reached its advanced stage (clinical stage IV), with clinical manifestations of distal metastases. In fact, survival rate has been found to be best in breast cancer patients without histological nodal involvement and worse in those with four or more lymph nodes showing evidence of metastatic disease (*Austoker et al., 1988*).

The mechanism by which benign or malignant breast cancer influences plasma RBP is unknown. RBP is known to show a high degree of sensitivity to an inadequate intake of protein, which may be due to the rapid turnover rate of RBP and its high requirement for tryptophan (*Rask et al., 1980*). The body weights of the patients participating in the present study indicated that the population was adequately nourished. Furthermore, there were no significant differences in the other plasma protein levels between the healthy controls and the patients. Both the total protein and the albumin/globulin ratios were found to be within the normal ranges in all groups of the study population, suggesting an adequate nutritional status. In patients with both benign and malignant breast diseases, vitamin A and its carrier proteins were decreased and the molar ratios of RBP/retinol were not different from the ratios in healthy subjects. This suggests that these two components of the retinol transport system were similarly affected in breast disease.

The present study has also evaluated some of the hospital patient data, such as body mass index; abnormal blood cholesterol levels; and use of adjuvant therapy for treatment of the disease, to further validate the results. Previously, breast cancer patients have been shown to be heavier, with a higher BMI (*Simard et al., 1990*). Additionally, breast cancer continues to be associated with dietary fat and high plasma lipid levels (*Howe et al., 1990; & Verreault et al., 1988*), and the effect of adjuvant treatment as reducing plasma vitamin A levels has also been recorded (*Schreurs et al., 1985*). These factors did not appear to have any appreciable effects in this study on the mean plasma values of vitamin A and its carrier proteins.

Benign breast disease is generally more prevalent amongst younger women compared to that of breast cancer (*Simard et al., 1990; & Weisburger, 1991*). This could explain the significant age difference found between the patients with metastatic breast cancer and benign breast disease (72.3 $\pm$ 3.7y versus 47.7 $\pm$ 1.8y, respectively), (mean $\pm$ SEM), participating in the present study. Despite the age difference the plasma RBP levels remained similar in both groups. It was of interest when the total population was divided into two groups according to age, it is the age group over 55 years i.e. post-menopausal that had a significantly higher mean level of plasma RPB than those under 55 years of age. It has been suggested (*Russell et al., 1988*), that there may be a reduction in RBP clearance from the plasma in older women. RBP is mainly

catabolized in the kidneys, although the formation of the RBP-prealbumin complex serves to reduce glomerular filtration and renal catabolism of RBP (*Goodman, 1984*). One may argue that this difference in RBP levels may be due to the difference in body weight between the age groups. Yet, when the benign breast disease and breast cancer patients were divided into the same age groups, this made no difference. Although age could be an important factor in influencing plasma vitamin A levels, the effect seems to be most pronounced only in early childhood, when liver stores of vitamin A can be as low as one-tenth that of the adult (*Wolf, 1990*).

Since zinc is involved in the synthesis of RBP (*Bates et al., 1981*), the plasma and tissue levels of zinc were also assessed. Plasma zinc is also a useful indicator of overall nutritional status, since marginal zinc deficiency is characterized by a slowing of physical growth (*Gibson, 1990*). The present study revealed no significant difference in plasma zinc concentrations between the breast cancer patients and the control subjects. These results are in agreement with others (*Kune et al., 1989*), who revealed no significant difference in serum levels of zinc between lung cancer cases and controls. This was true irrespective of the extent of the cancer or the cancer cell type. There are however, many studies which have demonstrated a negative association between reduced zinc or vitamin A levels in plasma and cancer in various sites including the esophagus (*Mellow et al., 1983*), and lung (*Atukorula et al., 1979*).

These contradictory findings may be explained by the fact that plasma zinc is subject to homeostatic control and capable of being maintained from an intracellular store (*Jackson, 1989*). The total amount of zinc in the adult is estimated to be 2g (*Fairweather-Tait, 1988*), and 10-20% of this amount is present in the plasma (*Gibson, 1990*). In persons with severe zinc deficiency, plasma zinc concentrations are usually low, but appear in the normal range for a marginal zinc deficiency. The plasma zinc concentration reflects changes in the size of the exchangeable zinc pool, but is also responsive to other metabolic changes (*King, 1990*).

The zinc content of tissue is believed to be a more reliable indicator of zinc status (*Meadows et al., 1981*). In the present study zinc concentrations were measured in the plasma, as well as in biopsy samples taken from a small sub-group of the patients with benign breast disease and post-operative disease free breast cancer participating in the study. This study revealed no significant difference between the values for tissue zinc concentrations between the two groups. Breast tissue is normally mixed with a considerable amount of adipose tissue, therefore the validity of the breast tissue concentration of zinc may be argued. However, Raukalahti et al. (1990), demonstrated from using the four anatomic quadrants of human breast that micro-nutrients are homogenously distributed in breast tissue and therefore not affected by the adipose tissue.

This supports the reliability of using breast tissue concentration of zinc as a reliable indicator of zinc status.

If the plasma and breast tissue levels of zinc are the reflections of true zinc status, then it could be assumed that the case-control differences in plasma retinol and RBP levels in the present study are due to neither zinc deficiency, inadequate protein status, or reduced RBP synthesis. Although both the benign breast disease and post-operative disease free breast cancer patients had significantly higher plasma zinc concentrations, the fact that this could be a reflection of dietary intake has not been determined from the study. Since the tissue zinc concentrations in the metastatic breast cancer patients and healthy controls were not measured, it cannot be concluded if the zinc concentrations were high or low.

RBP is synthesized in the liver and therefore it is known to be affected by the presence of disease (*Underwood, 1984*). In a subject with hepatic disorder, an impaired synthesis of RBP is a major contributing factor to low circulating levels of retinol. It is possible, therefore, that RBP levels in plasma could be affected in the presence of liver metastases. In the present study, the liver function tests (alkaline phosphatase, lactate dehydrogenase and serum glutamic oxaloacetic transaminase enzymes), were used as markers for hepatic malfunction and possible metastases. When the breast cancer patients were

divided according to normality of liver function, there was no significant difference found between normal and abnormal liver function.

Plasma RBP concentrations are known to be affected in catabolic states, post-surgery and hyperthyroidism (*Halpern, 1987*). Thus in burn patients, with the impact of stress and increased catabolism, vitamin A could play an important role in cellular multiplication and epithelial differentiation, since a post-burn decrease in vitamin A serum levels and its carrier proteins has been indicated (*Cynober et al., 1985*). It was unlikely that stress related to surgery had any effect in this study, since the period of blood collection since surgery made no significant difference. Similarly, these results are supported by another study (*Reichman et al., 1990*), which emphasized that the date of blood sample collection had no bearing on the results of low serum vitamin A levels and risk of prostate cancer. The decreased plasma RBP values in patients with breast disease, in parallel with lower plasma vitamin A levels may simply be a consequence of its impaired mobilization from the liver.

Plasma RBP levels appeared to be significantly lower in patients with metastatic breast cancer; benign breast disease; and the post-operative disease free cancer patients, compared with apparently healthy controls, In a similar study (*Basu et al., 1989*), involving 30 breast cancer patients with distal metastases, 29 patients with benign breast disease, and 30 healthy subjects, the serum RBP was found to be significantly affected only in the breast cancer

patients. Contrasted with the present study, these samples were obtained from a serum bank, collected for a different purpose and stored for many years. However, it is difficult to determine whether these findings are a result of a cause or an effect of the breast cancer disease process. Reduced RBP levels may be associated with an increased utilization of vitamin A. Cellular binding proteins of retinol and retinoic acid (for tissue uptake), have been shown to be associated with tumor cells, in comparison to the adjacent tissues (*Fex et al., 1985; Ikezaki et al., 1985; & Mehta et al., 1990*).

The underlying rationale for the lower plasma values of retinol and RBP in patients with breast disease cannot be explained, in that they do no appear to be a reflection of malnutrition as evident by normal plasma albumin levels. However, there is a relatively large body pool of albumin (3-5 g/kg body weight). Since albumin has a half life of 14-20 days, it is not very sensitive to short term changes in protein status (*Gibson, 1990*). In fact, as reported by Gibson (1990), the presence of a traumatic injury or ongoing stress could cause a transient fall in serum albumin and it may be artificially elevated in a state of semi-starvation. The half-life of RBP is about 12 hours and the body pool is small (2mg/kg body weight), therefore plasma RBP concentrations tend to fall rapidly in response to protein deprivation (*Shetty et al., 1979*). On the other hand prealbumin has a half-life of 2 days and a slightly larger body pool (10 mg/kg body weight, so it too is a sensitive index of protein status (*Gibson,*

1990). Prealbumin is also sensitive to minor stress and to deficiencies of vitamin A and zinc (*Gibson, 1990*). Plasma prealbumin levels were shown to be lower in the breast cancer patients in this study, although not statistically different from the controls.

Plasma RBP and prealbumin are known to be very sensitive to protein-energy malnutrition (*Ingenbleek et al., 1975; & Young et al., 1990*). Despite non-significant differences of plasma values for total protein, albumin, zinc and to a lesser extent prealbumin, it is possible that the lower levels of RBP are more sensitive to nutritional status and may be the reflection of subclinical malnutrition. Indeed, Shetty et al. (1979), has suggested that prealbumin and RBP could be used to detect subclinical malnutrition and monitor the effectiveness of dietary treatment. The significance of RBP may have some clinical application.

It appears from this study that RBP is a more sensitive reflection of individual protein status, than the other plasma proteins. An earlier study involving breast cancer patients (*Basu et al., 1988*), found that the plasma RBP levels were markedly reduced in two of the nine post-operative breast cancer patients, who had recurrence of the disease. It is noteworthy that in the present study the seven now deceased patients with metastatic breast cancer, had reduced levels of both retinol and RBP. Further, in a clinical study such as this, it is important to also emphasize individual values, since three patients

with metastatic breast cancer (now deceased), had seriously low levels of plasma RBP.

Since low levels of RBP would reduce the mobilization of retinol from the liver stores, making it unavailable to target tissues, it would seem that breast cancer is associated at least in part with the impaired metabolic availability of vitamin A. However, the results reveal that it is not only the biochemical status of vitamin A, but also the other factors involved in the metabolism of vitamin A which were affected. RBP was the main significant parameter changed in the fibrocystic disease and the breast cancer patients, which could be a reflection of the disease process or the protein status, which cannot be completely confirmed from this study. The reduced plasma RBP levels, however, did not appear to be specific only to a state of malignancy.

As the consistently lower RBP values in cancer patients could be a consequence of the cellular protein state, this acutely sensitive marker may be an important clinical tool. Measurement of this protein may provide an easier and more practical means for evaluating nutritional status and response to therapy (*Ota et al., 1985*). Since this is a prospective study, ideally the study population will also be followed further in the future, with regard to recurrence of the disease. Other research (*Knekt et al., 1990*), has indicated that the elevated risk of cancer among subjects with lower levels of retinol or RBP was mainly concentrated in the first 2 years of follow-up. The exact mechanism of

the role of retinol or RBP in cancer is still not completely understood. Breast cancer is a disease of abnormal differentiation of breast epithelial cells and retinoids could be considered possible agents with potential for arresting or preventing such cancer (*Osborne, 1991*), and therefore the binding proteins too, must play a critical role in facilitating their use.

4.1 CONCLUSION

The prevention and treatment of cancer continues to be one of the greatest challenges in medicine. Most of the evidence suggests that vitamin A may be protective against carcinogenesis (*Sporn et al., 1984*). Vitamins involved in the regulation of growth, differentiation and functioning of normal and malignant tissues, offer a new approach to cancer therapy (*Lopulescu, 1990*). More recent studies have shown that other vitamins, such as vitamin D, may also play a role in reducing breast cancer risk (*Garland et al., 1990*). When justified by sufficient definitive data from experimental and epidemiological investigations, intervention studies with microconstituents or foods rich in these substances can possibly be considered (*Nordevang et al., 1990*).

Breast cancer is a disease, of which the cause is still not fully understood and the primary prevention of breast cancer remains an ideal. As implicated from this study, the development of intermediate markers for breast cancer is an important research priority. From an analysis of the results of this study

and other supporting studies (*Basu et al., 1989*), it is possible that RBP being so highly sensitive to both a reduced retinol and protein status could be such a diagnostic or prognostic tool. The possible prognostic significance of RBP cannot be eliminated and could be substantiated with further research.

The major findings of this study were that plasma retinol and specifically RBP levels were lower in all groups associated with breast disease compared to the healthy controls. It is also the metastatic breast cancer patients, furthest advanced in the disease process that had the lowest plasma values for RBP, prealbumin and zinc. Plasma levels of retinol did not appear to be as sensitive as the other biochemical parameters (specifically RBP), to the reduced vitamin A status in breast disease. However, a mechanism still cannot be identified for the cause of the reduced plasma RBP levels in benign breast disease and breast cancer patients.

There is certainly a need to identify a biological marker, as is the case of reducing myocardial infarction or atherosclerosis with specific intervention, as a result of using blood cholesterol (or lipoprotein) levels as an intermediate marker. Reliable diet survey methodologies are also needed in epidemiologic studies to establish pre-disease intake patterns (*Freudenheim et al., 1988*). At present, advocating a reduction in dietary fat represents one opportunity for disease prevention in breast cancer (*Boyar et al., 1988*). Implications from this

study reveal the importance of RBP being used as an early diagnostic indicator for epithelial cancers.

As a result of advanced biochemical screening techniques such as the analysis of RBP, this could be applied to improving the survival rate from breast cancer. By identifying persons with increased risk of the disease an appropriate prevention program could be conducted, or the risk of breast cancer within the population could be decreased with suitable nutritional intervention (*Iacono, 1987*). Yet, this may not aid in predicting long-term survival rates, as the discriminant analysis revealed only a small proportion of the study population being correctly classified according to plasma retinol, RBP and prealbumin levels.

It is suggested that an extension of this study would be a follow-up of the patients (for at least 5 years), on the recurrence of the disease in the study population. If fibrocystic breast disease is a predisposing factor for breast carcinoma, it is important to conduct a follow-up study to see if the benign breast disease patients develop malignant breast disease and to determine the diagnostic significance of RBP. Similar studies could also be completed, which involve assessing dietary intakes of the patients and correlations between other biochemical indices, such as plasma triglyceride and cholesterol levels. Clinical trials where supplementation with retinoids as a preventative measure, in cases of low circulating levels of vitamin A, could possibly be investigated. Future

research should prove extremely exciting and provide new information which could markedly reduce the incidence of breast cancer. One of the major tools for the prevention of cancer could indeed be the manipulation of micro-nutrients in our diet.

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APPENDICES
(#1-11)

APPENDIX #1
DEFINITIONS ASSOCIATED WITH BREAST CANCER

Benign Breast Disease (Chronic Cystic Mastitis):
Originates from proliferation of epithelial and connective tissue, with fibrocystic (FD) changes and fibroadenoma being most frequent. An unusually dense, relatively acellular fibrous stroma has been termed fibrous mastopathy.

Biopsy:
Sample aspiration by fine-needle or operative open technique, with incision (removal of part of tissue), or excision (removal of whole tissue concerned).

Cancer:
Abnormal, uncontrolled growth of a lump or a mass that also destroys normal tissue.

Carcinoma:
A form of cancer involving epithelial tissue and coverings of internal and external surfaces, including the breast.

Fibroadenoma:
Stromal proliferations of greater magnitude which are treated by simple excision without the likelihood of recurrence, unless multiple. The degree of cellularity in the stroma varies, but is always benign.

Fibrocystic Disease (FD):
Is applied to any condition that on microscopic examination gives the impression of an abnormal, but not malignant state.

Infiltrating Ductal Carcinoma:
The most likely site of origin of breast adenocarinoma (40-60 percent of cases), is the terminal ductal site (with nuclear morphology described as well-differentiated, moderately differentiated and poorly differentiated).

Infiltrating Lobular Carcinoma:
Characteristically permeates a desmoplastic stroma in a linear fashion (with a reported frequency of 5-10 percent of breast carcinoma). The tumors vary from clinically inapparent, microscopic lesions to covering the entire breast with a poorly defined lesion.

Infiltrating/Non-Infiltrating Staging of Breast Carcinoma:

Stage I	non-infiltrating: (non-invasive)	no known metastases
Stage II	infiltrating: (invasive)	no known metastases some nodal involvement
Stage III	infiltrating: (invasive)	possible metastases nodal involvement
Stage IV	infiltrating: (invasive)	distant metastases

Mastectomy:

Is the surgery involved in the removal of infected breast tissue (in ascending order of severity):

*segmental or partial mastectomy/lumpectomy
*total/simple mastectomy, without lymph nodes
*modified radical or total/simple mastectomy, with lymph nodes
*radical mastectomy, with pectoralis major (muscle)
*extended radical mastectomy (regional or distant sites)

Medullary Carcinoma:

Characteristically large size of tumor mass and histologically poorly differentiated; yet patients with medullary carcinoma have a more favourable prognosis, compared to individuals with common adenocarcinoma of the breast.

Metastasis:

A transfer of carcinoma, from one organ to another that is not directly connected with it.

Papillary Carcinoma:

Generally small carcinomas (rarely larger than 2-3 cm in maximal diameter), well circumscribed and accounts for less than 2 percent of breast carcinoma (often present later in life).

(*Re: Austoker et al., 1988; Carter 1990; Escott-Stump, 1988; Kubli et al, 1989; Vorherr, 1980; & Yeatman et al., 1991*).

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"A mission of progressive health care"

April 19th, 1991

Dr. T.K. Basu, PhD, FICN, FACN
Department of Foods and Nutrition
Faculty of Home Economics
University of Alberta
308 Home Economics Building
Edmonton, Alberta
T6G 2M8

Dear Dr. Basu:

Re: Research Program
Retinol-A Protein Study in Breast Diseases

This letter is to officially advise you that the Board and Administration is committed to the above study and has agreed to participate fully with providing details and other support as required. Dr. M. Ray and other members of our staff are authorized to participate in the research program and information regarding the above will be shared accordingly (includes the submission of specimens for the study, medical records information and review of miscellaneous patient files).

I trust that this is satisfactory and hope that the information obtained from the Archer Memorial Hospital will add to the overall benefit of this study. We look forward to hearing more about the final outcome of this study.

Yours very truly,

Harold James,
Executive Director

HJ/rk

Appendix #2

APPENDIX #3 BREAST CANCER PATIENT CASES - METASTATIC (TOTAL 17)

SUBJECT #	AGE (in 1991)	MENO-PAUSAL STATE (at surgery)	FAMILY HISTORY (of Breast Cancer)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$	BREAST SURGERY	DATE (of surgery)	ADJU-VANT THERAPY	LIVER FUNCTION TESTS	STAGE DISEASE
L7143	84	post-m.	ϕ	58	1.55	24.2	R-TM	Nov.'86	HORM.	H-Chol.	IDC (II/III)
L7140	52	pre-m.	ϕ	65	1.70	22.5	R + SM R-MRM	Jan.'83 Feb.'83	CHEM. RAD. HORM.	H-LDH	IDC (II/III)
L564	65	post-m.	ϕ	81	1.60	28.0	L-SM R-EB	Aug.'87	RAD.	H-T.Bili	IDC (III)
L7074	91	post-m.	ϕ	44.5	1.55	18.5	L-SM	Jan.'82	HORM.	Norm.	IDC (III) + D
L7192	45	post-m.	ϕ	83	1.60	32.4	R-EB R-MRM	Dec.'87 Aug.'89	CHEM. RAD.	Norm.	IDC (III)
L7073	84	post-m.	ϕ	52	1.65	19.1	R-SM	Feb.'88	HORM.	Norm.	IDC (III) + D
L7075	82	post-m.	ϕ	74.7	1.63	28.1	L-SM L-TM	Sept.'88 Aug.'89	ϕ	Norm.	IDC (III)
L7047	80	post-m.	ϕ	72	1.57	29.2	R-EB R-MRM	Jan.'82	ϕ	Norm.	IDC (III/IV)
L3860	70	post-m.	ϕ	61.7	1.55	25.7	L-MRM	Sept.'88	CHEM. HORM.	Norm.	IDC (III/IV) + D

APPENDIX #3 (CONTINUED) BREAST CANCER PATIENT CASES - METASTATIC (M) (TOTAL 17)

SUBJECT #	AGE (in 1991)	MENO-PAUSAL STOfE (of surgery)	FAMILY HISTORY (of Breast Cancer)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$	BREAST SURGERY	DATE (of surgery)	ADJU-VANT THERAPY	LIVER FUNCTION TESTS	STAGE DISEASE
L3844	75	post-m.	FH	94.3	1.52	40.8	R-EB R-MRM	May'83	CHEM. RAD. HORM.	Norm.	IDC (III) + D
L3969	87	post-m.	ϕ	88.5	1.63	33.3	ϕ	ϕ	ϕ	Norm.	Adv.BC (III/IV)
L3968	90	post-m.	FH	67	1.52	29.0	R-NB	April'84	ϕ	Norm.	Adv.BC (III/IV)
B50	80	post-m.	ϕ	59	1.55	24.6	R-MRM	'75	CHEM. RAD.	N/A	IDC (IV) Met.+ D
B51	71	post-m.	ϕ	54	1.50	24.0	R-MRM	'86	RAD.	H-T.Bili. H-Alk.Ph.	IDC (IV) Met.+ D
B52	58	pre-m.	ϕ	68.1	1.65	25.0	L-TM	'85	CHEM. RAD.	H-Alk.Ph. H-SGOT	IDC (IV) Met.+ D
B6430	74	post-m.	ϕ	85	1.57	34.5	L-TM	June'80	CHEM.	H-LDH	IDC (IV) + Met.
B322	42	pre-m.	ϕ	67.3	1.60	26.3	L-TM	Oct.'90	CHEM. RAD.	Norm.	IDC (III) + Met.

APPENDIX #4 BREAST CANCER PATIENT CASES - POST-OPERATIVE DISEASE FREE (TOTAL 25)

SUBJECT #	AGE (in 1991)	MENO-PAUSAL STATE (at surgery)	FAMILY HISTORY (BC)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$	BREAST SURGERY	DATE (of surgery)	ADJUNCT-IVE THERAPY	LIVER FUNCTION	STAGE DISEASE
L7046	54	post-m.	ϕ	77	1.55	32.0	R-SM R-MRM	Feb.'89	ϕ	Norm.	MC (I stage)
L7048	77	post-m.	FH	74	1.52	32.0	R - EB R - MRM	Nov.'88 Dec.'88	CHEM. RAD. HORM.	H-Chol.	IDC (I)
L4131 + tissue	63	post-m.	ϕ	59	1.52	25.5	R - EB R - MRM	Nov.'89	ϕ	H-Chol.	ILC (II)
L7070	62	post-m.	ϕ	67.5	1.68	23.9	L - EB L - MRM	July'87	ϕ	H-Chol.	IDC (I)
L1080	33	post-m.	FH	56	1.63	21.1	R-EB R-MRM	May'89	CHEM.	H-SGOT	IDC (I)
L6890 + tissue	89	post-m.	FH	60.2	1.65	22.1	R-SM	Feb.'90	ϕ	Norm.	IDC (II)
L287 + tissue	48	pre-m.	ϕ	78.2	1.70	27.1	R - EB R - MRM L - EB	Feb. '87 March '87 Oct.'87	CHEM. RAD.	H-Chol.	FD + IDC (I)
L517 + tissue	77	post-m.	ϕ	65	1.66	23.6	R-MRM L-SM	Feb.'90	HORM.	Norm.	IDC (I)
B6338	66	post-m.	ϕ	65.9	1.63	24.8	R-TM	Nov.'88	ϕ	Norm.	ILC (II)
B2067	58	pre-m.	ϕ	74.3	1.59	29.4	R - TM	'73	ϕ	Norm.	IDC (I)
B406	66	post-m.	ϕ	109.2	1.64	40.6	R-SM	Dec.'88	RAD.	H-Alk.Ph.	MC (II)

APPENDIX #4 (CONTINUED) BREAST CANCER PATIENT CASES - POST-OPERATIVE DISEASE FREE (TOTAL 25)

SUBJECT #	AGE (in 1991)	MENO-PAUSAL STATE (at surgery)	FAMILY HISTORY (BC)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$	BREAST SURGERY	DATE (of surgery)	ADJUNCT-IVE THERAPY	LIVER FUNCTION	STAGE DISEASE
B169	83	post-m.	ϕ	78.2	1.54	33.0	R + L - TM	Nov.'89	HORM.	H-Alk.Ph.	R-ILC (II) L-IDC (II)
B195	78	post-m.	ϕ	81.82	1.53	35.0	R-IB	Jan.'91	ϕ	Norm.	IDC (II/III)
B347	95	post-m.	ϕ	45.27	1.52	19.6	R - TM	May'90	ϕ	Norm.	NIDC (II)
B223	79	post-m.	ϕ	51.5	1.49	23.2	R - SM L - SM	'75 '79	RAD.	H-Alk.Ph.	IDC (I)
B135	64	pre-m.	ϕ	72.73	1.60	28.3	L-TM	Dec.'76	ϕ	Norm.	IDC (II)
B100	81	post-m.	ϕ	53.73	1.65	21.2	L-SM	'79	RAD.	H-T.Bili. H-SGOT	IDC (II)
B244	49	pre-m.	ϕ	71	1.57	28.8	R-MRM	Sept.'85	RAD.	Norm.	ILC (I)
B95	88	pre-m.	ϕ	45.45	1.51	19.9	R-SM	Feb.'85	ϕ	H-Alk.Ph.	IDC (I)
B721	58	post-m.	ϕ	63	1.60	24.5	L-TM	Oct.'90	HORM.	H-Alk.Ph.	IDC (II)
B96	63	post-m.	ϕ	56.7	1.60	22.1	R-SM	May'79	RAD.	Norm.	NID/LC (I)
B1861	70	post-m.	ϕ	84.2	1.64	31.3	L-MRM	Mar.'91	RAD.	Norm.	IDC (II/III)
B485	75	post-m.	ϕ	76.5	1.60	29.8	L-SM	Oct.'90	RAD. HORM.	Norm.	IDC (II)

APPENDIX #4 (CONTINUED) BREAST CANCER PATIENT CASES - POST-OPERATIVE DISEASE FREE (TOTAL 25)

SUBJECT #	AGE (in 1991)	MENO-PAUSAL STATE (at surgery)	FAMILY HISTORY (BC)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$	BREAST SURGERY	DATE (of surgery)	ADJUNCT-IVE THERAPY	LIVER FUNCTION	STAGE DISEASE
B3043	54	pre-m.	ϕ	70.1	1.74	23.2	L-TM	Aug.'83	ϕ	Norm.	DC (I)
B992	63	post-m.	ϕ	73.6	1.54	31.0	R-TM	Oct.'87	RAD.	Norm.	IDC (II)

APPENDIX #5
PATIENT CASES - BENIGN BREAST DISEASE (TOTAL 28)

SUBJECT #	AGE (in 1991)	MENOPAUSAL STATE (at surgery)	FAMILY HISTORY (BC)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$	BREAST SURGERY	DATE (of surgery/ diagnosis)	STAGE DISEASE
L225	40	pre-m.	ϕ	52.5	1.58	21.0	R-EB	Nov.'87	FD
L224	44	post-m.	ϕ	70	1.55	29.1	R-EB	Oct.'88	FD
L131	41	pre-m.	ϕ	57.5	1.55	23.9	R-EB	Aug.'88	FD
L263	50	pre-m.	ϕ	59	1.58	23.63	R-EB L-NB	April'84 April'86	FD
L264	39	pre-m.	ϕ	46	1.50	20.5	L-EB	March'88	FD + D
L657	29	pre-m.	ϕ	58	1.65	21.3	L-EB	April'91	FD
L262 + tissue	69	post-m.	FH	56.5	1.60	22.07	R-EB L-EB L-EB R-EB	Aug.'87 Aug.'88 Oct.'89 March'90	FD
L476 + tissue	51	post-m.	ϕ	78	1.63	29.4	L-IB	Nov.'89	FD
L404 + tissue	58	post-m.	ϕ	52	1.63	19.5	ϕ	Jan.'89	Lipoma
L407 + tissue	44	pre-m.	ϕ	97	1.68	24.4	L-EB R-NB	April'90	FD
L5555 + tissue	36	pre-m.	ϕ	50	1.58	20.0	R-SM	Jan.'91	FA
L5833 + tissue	57	post-m.	FH	61	1.62	23.2	L-SM	Jan.'91	FD

APPENDIX #5 (CONTINUED)
PATIENT CASES - BENIGN BREAST DISEASE (TOTAL 28)

SUBJECT #	AGE (in 1991)	MENOPAUSAL STATE (at surgery)	FAMILY HISTORY (BC)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$	BREAST CANCER	DATE (of surgery/ diagnosis)	STAGE DISEASE
L1404 + tissue	44	post-m.	ϕ	70	1.73	23.4	R-EB	June'90	FD
B34	45	pre-m.	ϕ	58	1.53	24.7	L-NB R-EB	Dec.'87	FD
B375	39	pre-m.	ϕ	56.2	1.57	23.4	L-EB	Nov.'90	FD
B199	49	post-m.	ϕ	59.4	1.70	20.6	R-NB + R-EB	July'86	FD
B243	25	pre-m.	ϕ	67.6	1.63	25.5	R-EB	Oct.'90	FA
B2241	52	pre-m.	ϕ	63.6	1.73	21.3	R-NB	June'86	FD + R*
B249	47	pre-m.	ϕ	63.2	N/A	N/A	R + L - NB	March'87	FD
B297	47	pre-m.	ϕ	53	1.56	21.8	R + L - NB	Oct.'90	FD
B157	55	pre-m.	ϕ	59.6	1.60	23.3	R - NB	April'86	FD
B70	51	pre-m.	ϕ	57.4	1.60	22.4	ϕ	Dec.'90	FD
B371	54	pre-m.	ϕ	56.6	1.74	18.7	R-EB	June'88	FD + Lipoma
B2251	59	post-m.	ϕ	65	1.69	22.8	R + L - NB + EB	June'84	FD
B8337	52	post-m.	ϕ	77.3	1.68	27.4	L - NB	Aug.'87	FD
B2231	47	pre-m.	ϕ	60.5	1.63	22.6	R + L - OB + NB	Oct.'80	FD

APPENDIX #5 (CONTINUED)
PATIENT CASES - BENIGN BREAST DISEASE (TOTAL 28)

SUBJECT #	AGE (in 1991)	MENOPAUSAL STATE (at surgery)	FAMILY HISTORY (BC)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$	BREAST CANCER	DATE (of surgery/ diagnosis)	STAGE DISEASE
B2177	59	post-m.	ϕ	85.9	1.68	30.4	R-OB	March'91	FA
B3131	49	pre-m.	ϕ	94.7	1.59	37.5	R + L OB + NB	Oct.'80	FD
L7 + tissue only	33	pre-m.	FH	44	1.59	17.4	R-EB	Jan.'90	FD
L381 + tissue only	43	post-m.	ϕ	86.8	1.68	30.8	R-EB L-SM L-EB R-EB R+L-EB	Oct.'86 Oct.'88 May'89 Oct.'89 Feb.'91	FD

APPENDIX #6 HEALTHY CONTROLS - POST-MENOPAUSAL SUBJECTS (TOTAL 25)

SUBJECT #	AGE (in 1991)	WEIGHT (kg)	HEIGHT (m)	BMI: $\frac{kg}{m^2}$
60	73	70	1.53	30.3
61	78	67.5	1.71	23.1
62	70	74.7	1.56	30.7
63	77	79.2	1.56	32.6
64	75	49.5	1.53	21.4
65	71	80.5	1.61	31.1
66	74	73.3	1.61	28.3
68	75	48.2	1.54	20.3
69	78	79.1	1.70	27.4
76	66	60.9	1.47	28.3
78	73	61.8	1.48	28.4
21	82	70.7	1.65	25.9
24	71	77.3	1.63	29.1
26	71	68.4	1.61	26.4
27	72	72	1.57	29.2
29	69	63	1.68	22.3
30	82	76.8	1.57	31.2
31	70	75.7	1.65	27.8
32	73	49.9	1.50	22.2
33	74	53.2	0.87	22.2
35	71	62	1.55	25.8
36	80	61.7	1.50	27.4
37	78	61.9	1.60	24.2
38	74	65	1.66	23.6
39	75	63.6	1.63	23.9

APPENDIX #7A
KEY TO TABLE ABBREVIATIONS

Adv.BC = Advanced Breast Cancer
Alk.Ph. = Alkaline Phosphatase
BC = Breast Cancer
CHEM. = Chemo-Therapy
Chol. = Cholesterol
D = Deceased
E.BC = Early Breast Cancer
FA = Fibroadenoma
FD = Fibroystic Disease
FH = Family History
EB = Excisional Biopsy
H = Abnormal Level (High)
HORM. = Hormone Therapy
IB = Incisional Biopsy/Lumpectomy
IDC = Infiltrating Duct Carcinoma
ILC = Infiltrating Lobular Carcinoma
L = Left Breast
LDH = Lactate Dehydrogenase
M = Malignant
M + DF = Malignant + Disease Free
Mam. = Mammography
MC = Medullary Carcinoma
Met. = Metastases
MRM = Modified Radical Mastectomy
N/A = Not Available
NB = Needle Biopsy
NIDC = Non-Infiltrating Duct Carcinoma
NILC = Non-Infiltrating Lobular Carcinoma
Norm. = Normal Level
PC = Papillary Carcinoma
Pre-m. = Pre-menopausal
Post-m. = Post-menopausal
R* = Reccurence
R = Right Breast
RAD. = Radiation Treatment
SM = Segmental Mastectomy/Lumpectomy
SGOT = Serum Glutamic Oxaloacetic Transaminase (AST)
T. Bili. = Total Bilirubin
TM = Total Mastectomy

APPENDIX #7B
NORMAL RANGES FOR LIVER FUNCTION TESTS

Alk.Ph. = 15-65 U/L
Chol. = < 6.5 mmol/L
LDH = 110-190 U/L
SGOT = 7-35 U/L
T.Bili. = 3-20 µmol/L

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APPROVAL

FOR

PROPOSAL ON HUMAN RESEARCH

This is to certify that **Dr. Tapan Basu**

a staff member in the **Department of Foods and Nutrition**

submitted a proposal for a research project entitled:

Significance of retinol-binding protein in breast cancer

to the Faculty of Home Economics Ethical Review Committee. The ethical criteria for human research have been met.

Date: May 15, 1991

Dr. T. Clandinin, Chair

Clandinin\Ethics.mst

Appendix #8

APPENDIX #9
STATISTICAL DESIGN - DETERMINATION OF SAMPLE SIZE

The following formula is used to calculate sample size:

$$\frac{n\text{-}z^2s^2}{d^2}$$

where n = sample size
z = reliability coefficient
s = standard deviation
d = interval width

i) **RBP:** **Assuming normal level of RBP is 5 mg/dL with a standard deviation say of 1 mg/dL. In the disease state, we may expect say a 1mg/dl drop in plasma level of RBP, therefore d = 1/2 = 0.5 mg/dL in either direction.**

If z = 1.96 (for 0.95 confidence coefficient)

$$n \text{-} \frac{(1.96)^2\ (1)^2}{(0.5)^2} = 16$$

Therefore, suggest at least 20 subjects for each group to be compared with the expectation that the effect will be at least 30% of the statistical comparison.

ii) **VITAMIN A:** **Assuming normal levels of vitamin A is 75 μ/dL with a standard deviation say of 10 μ/dL. In the disease state, we may expect say a 10 μ/dL drop in plasma level of vitamin A, therefore d = 10/2 = 5 μg/dL in either direction.**

If z = 1.96 (for 0.95 confidence coefficient)

$$n \text{-} \frac{(1.96)^2\ (1)^2}{(0.5)^2} = 16$$

Also suggests a minimum of 16 subjects.

NOTE: **According to the use of power tables** (*Cohen, 1977*), the minimum sample size for each group is confirmed as 17.

APPENDIX #10
PATIENT DATA FORM

RESEARCH RELATED TO BREAST CANCER

HOSPITAL: **DOCTOR:**

Patient #:

Birthdate:

HISTORY:

1. **Date of diagnosis:**

2. **Type of diagnosis (details):**

3. **Stage of the disease at the time of blood/tissue collection:**

4. **Date of blood/tissue collection:**

5. **Treatment prior to blood collection:**

6. **Prognosis (current):**

7. **Additional comments:**

APPENDIX #11
APPROXIMATE ZINC CONTENT OF MAJOR ORGANS AND TISSUES IN THE NORMAL ADULT (70 kg Man)

TISSUE	APPROXIMATE ZINC CONCENTRATION (μmol/g)	TOTAL ZINC CONTENT (g)	PROPORTION OF TOTAL BODY ZINC (%)
Skeletal Muscle	7.8	1.53	57 (approx.)
Bone	15.3	0.77	29
Skin	4.9	0.16	6
Liver	8.9	0.13	5
Brain	1.7	0.04	1.5
Kidneys	8.4	0.02	0.7
Heart	3.5	0.01	0.4
Hair	23.0	<0.01	0.1 (approx.)
Blood Plasma	0.2	<0.01	0.1 (approx.)

Table adapted from *Jackson*, 1989